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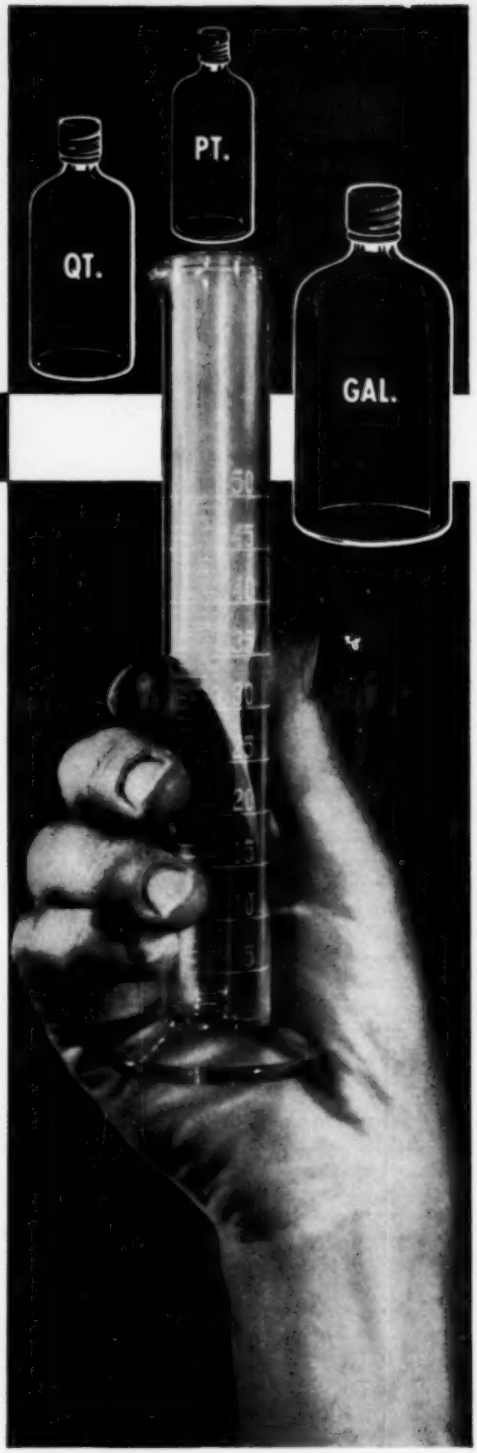

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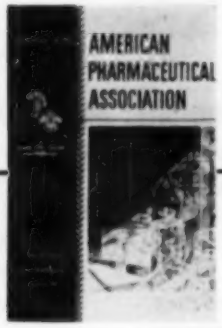

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Look for details in the Practical Pharmacy Edition of This Journal—Out February 20

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JOURNAL OF THE AMERICAN PHARMACEUTICAL ASSOCIATION

VOLUME XLIII | *FEBRUARY, 1954* | NUMBER 2, CONSECUTIVE NO. 4

The Effect of Gamma Radiation on Some Pharmaceutical Products*

By J. CONTROULIS,† C. A. LAWRENCE,‡ and L. E. BROWNELL§

A number of pharmaceuticals in finished product form have been subjected to irradiation by gamma rays emanating from a cobalt-60 source. Sterilization of purposely contaminated samples of the pharmaceuticals was effected successfully in all of the products studied. In addition, uncontaminated samples were irradiated under similar conditions to determine possible side effects of the gamma radiation.

GAMMA RADIATION from a cobalt-60 source has been used to sterilize a variety of pharmaceutical products. The results indicate success of the method on an experimental basis and point the way to larger-scale industrial application of this technique.

Pharmaceutical products used for parenteral therapy must be sterile. Sterilization with heat is usually convenient with products that are not heat-labile. Heat-labile products can usually be sterilized by microfiltration and by maintaining sterility during packaging of the product in sterile areas. However, a number of therapeutic substances resist the successful application of either of these techniques. Furthermore, sterilization of the finished package would assure the potential user of the utmost safety with regard to possible contamination of the parenteral product. In this respect, sterilization by ionizing radiation offers some interesting possibilities. Others (1, 2) have reported the use of electron bombardment as a means of sterilization. The authors wish to report some tests made with gamma radiation from a kilocurie cobalt-60 source of the type described by Manowitz (3).

PROCEDURE

Figure 1 is a sectional view of the container for the Michigan kilocurie source. A pneumatic lift is used for charging and removing the 1 3/8 inch (diameter) aluminum tube in which the samples are placed for irradiation.

It has been found that these kilocurie sources vary somewhat in their strength and it is necessary to calibrate each source separately. A calibration of the Michigan kilocurie source gave a value of 79,000 rep (in air) as of July 1, 1952 (4). One rep represents an energy absorption dose of 83.8 ergs per gram in air or about 3.6×10^{-6} B. t. u. per pound.

The tests of the effects of gamma radiation on some pharmaceutical products were the subject of cooperative research between the Fission Products Laboratory of the Engineering Research Institute[1]

* Received September 24, 1953, from Parke, Davis & Co., Detroit, Mich.

† Parke, Davis & Co., Detroit, Mich.

‡ Formerly Assistant Professor of Bacteriology, Medical School, University of Michigan, Supervisor, Michigan Memorial Phoenix Project 20 and Bacteriological Experiments of Michigan Memorial Phoenix Project 41. At present: Director, Bureau of Laboratories, County of Los Angeles Health Dept., 241 North Figueroa Street, Los Angeles 12, Calif.

§ Associate Professor of Chemical Engineering; Director, Fission Products Laboratory, University of Michigan and Food Experiments of Michigan Memorial Phoenix Project 41.

[1] The Atomic Energy Commission has contracted with various universities, including the Engineering Research Institute of the University of Michigan, to investigate possible industrial uses of radioactive waste fission products. As a result of this contract (No. AT(11-1)-162) the Fission Products Laboratory was established and equipped with a kilocurie cobalt-60 source obtained from the Brookhaven National Laboratory.

at the University of Michigan, Parke, Davis & Company of Detroit and the Michigan Memorial Phoenix Project.[1]

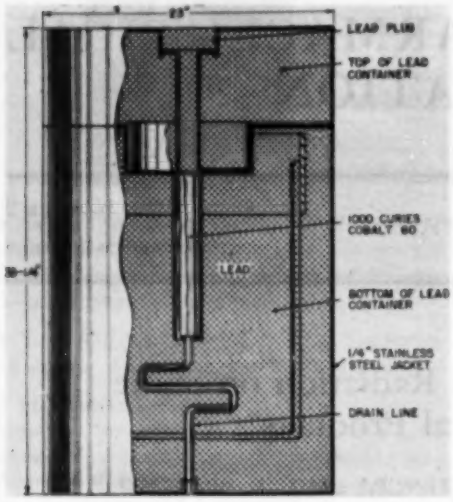

Fig. 1.—Sectional view of the container for the Michigan kilocurie sources.

In some preliminary cooperative studies made by the Fission Products Laboratory and Parke, Davis & Company, a variety of pharmaceuticals including antitoxins, hormones, and vitamins were irradiated and assayed. Three samples of each product were contaminated with *B. subtilis* and reserved as controls. Another group of three samples of each product were contaminated and irradiated for one hour. A third group of three samples of each product were contaminated and irradiated for twenty-four hours. A similar set of three groups of three samples of each product were not contaminated, but one group was left as control, another for one-hour irradiation, and the third for twenty-four hour irradiation. The contaminated set of samples was used for sterility evaluation and the uncontaminated set for the biological activity evaluation.

The uncontaminated irradiated and uncontaminated control samples were submitted for various assays to sections within Parke, Davis & Company to ascertain whether or not any deterioration of the pharmaceutical had occurred as a result of irradiation. The methods of assaying the various products were as close as possible to the accepted assay procedures regularly used in manufacturing.

Calcium gluconate samples were observed visually to detect any breakdown of the solution. Ascorbic acid samples were tested colorimetrically. Pitocin[2] samples were assayed for oxytoxic activity. Theelin[4] samples were assayed via ultraviolet absorption spectrum to determine any structural change in the estrone molecule. Tetanus antitoxin samples were assayed biologically for tetanus units. Diphtheria-tetanus-pertussis samples were assayed via pertussis agglutination and also for tetanus-combining power. The contaminated irradiated and control samples were tested for sterility.

The results of the activity assays and the sterility assays appear in Table I.

From the tabulated data it is seen that gamma radiation accomplished sterilization in all products receiving a dosage of 2.04 million rep (in air) in twenty-four hours. While there may have been a statistically important reduction in the number of viable organisms after one hour of exposure to a radiation dose of 85,000 rep (in air), there were always enough organisms present to render the samples nonsterile at this lower dosage. As to the effects of the irradiation upon biological activity the following observations can be made.

No. 1—There appears to be no detrimental effect on the 10% calcium gluconate solution. (The evaluation was based on the visible appearance of the solution; turbidity, amorphous deposit, or crystalline growth are the signs of deterioration usually noted with this product.)

No. 2—The assays of irradiated ascorbic acid solutions show no statistical departure from that of the unirradiated sample.

No. 3—While there is a decrease in Theelin content of the irradiated samples compared to the control, the lower values are still above the label claim.

No. 4—Pitocin is the most clear-cut example of a pharmaceutical product whose potency is drastically affected by gamma radiation.

No. 5—Since the triple combination of diphtheria-tetanus-pertussis is a difficult product to assay, pertussis agglutination and tetanus-combining power were used to determine the effect of the irradiation on these components. While the irradiated samples show the same response as the unirradiated controls, the value for tetanus-combining power of the control is much lower than it should have been. Therefore, this evaluation is probably not a valid one and, in future studies, this point will be considered further.

No. 6—In the case of the tetanus antitoxin, the radiation dosage used for sterilization causes appreciable deterioration of the product.

No. 7—Antuitrin G can be sterilized by gamma irradiation but the effect of the radiation on the potency is not yet known. However, it is of significance to note that the irradiation produced sterilization even though the product was in the dry state.

Figure 2 is a reproduction of a photograph of some of the irradiated samples and the controls. Irradiation for one hour (85,000 rep in air) caused a slight but definite tinting of the glass. This discoloration is sufficient to be recorded by a camera when samples irradiated for one hour were photographed besides unirradiated samples. In the case of the samples receiving a dose of twenty-four hours of radiation (2.04 million rep in air) the discoloration was severe.

[1] This project is a memorial to the Michigan dead of World War II. The Regents of the University at their meeting on May 1, 1948, on recommendation of their War Memorial Committee, voted to "create a War Memorial Center to explore the ways and means by which the potentialities of atomic energy may become a beneficial influence in the life of man, to be known as the Phoenix Project of the University of Michigan."

[2] Trademark of Parke, Davis & Company for oxytocic fraction of pituitary gland.

[4] Trademark of Parke, Davis & Company for estrone.

TABLE I.—RESULTS OF GAMMA RAY STERILIZATION

Sample No. and Product	Tests	Potency of Uncontaminated Samples, Irradiation Time 0 Hr.	1 Hr.	24 Hr.	Sterility of Contaminated Samples, Irradiation Time 0 Hr.	1 Hr.	24 Hr.
1. Amp. No. 239 calcium gluconate	Activity	OK	OK	OK	..	..	..
	Sterility	...	...	...	C[a]	C	S[b]
2. Amp. No. 315 ascorbic acid	Activity, mg./ml.	275.5	283.3	273.0	..	..	..
	Sterility	...	...	...	C	C	S
3. Amp. No. 304 Theelin aq. suspension	Activity, mg./ml.	2.70	2.21	2.19	..	..	..
	Sterility	...	...	...	C	C	S
4. Amp. No. 160 Pitocin	Activity	100%	50%	0.02%	..	..	..
	Sterility	...	...	...	C	C	S
5. Biol. No. 2091 diph. tet. pert. combination	Agglutination tetanus combining power, u./ml.	0.8	0.8	0.8	..	..	..
	Sterility	...	...	...	C	C	S
6. Tetanus antitoxin	Activity, u./ml.	1900	1900	1500	..	..	..
	Sterility	...	...	...	C	C	S
7. Antuitrin[c] growth	Activity	...	...	...	..	..	..
	Sterility	...	...	...	C	C	S

[a] Contaminated.
[b] Sterile.
[c] Trademark registered by Parke, Davis & Company for anterior pituitary gland fraction.

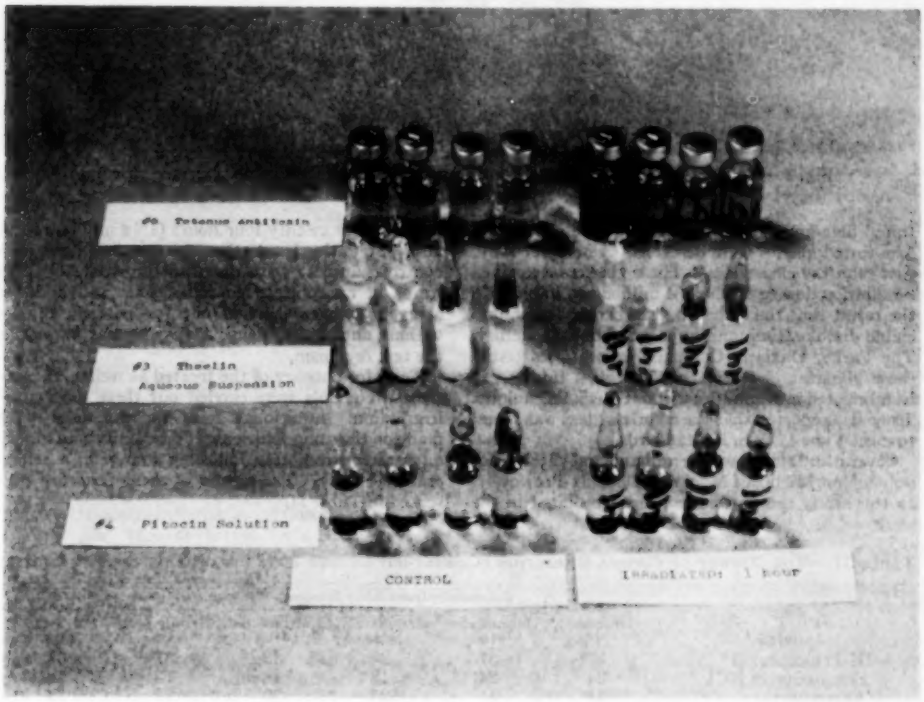

Fig. 2.—Irradiated samples (one hour) and controls of some pharmaceuticals.

as shown in Fig. 3. The glass darkened to a deep amber or brown color.

The discoloration of the glass is considered to be one of the real disadvantages of radiation sterilization. Thus, it appeared desirable to approach the problem of radiation sterilization of pharmaceutical products by a search for specimens (types) of glass which would resist radiation discoloration. Samples of three such glasses were received[b] and examined: the first, a colorless glass; the second, a lightly

[b] Our appreciation is due to Dr. H. P. Hood of the Corning Glass Works for supplying these samples of glass.

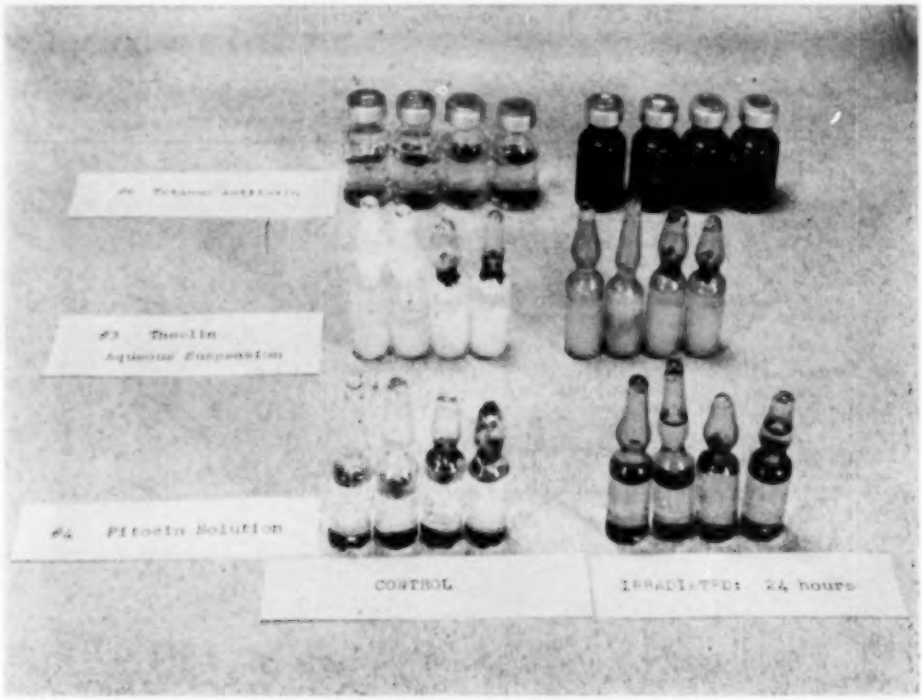

Fig. 3.—Irradiated samples (twenty-four hours) and controls of some pharmaceuticals.

tinted blue-green; and the third, a pale olive color. The tinted glasses were described as being of lower cost than the clear glass. These glasses were given a radiation dosage of about 4 million rep in air with the result that the colorless glass showed no appreciable discoloration, and appears to be satisfactory. The Parke, Davis & Company laboratories tested these glasses and found that the glass tubing can be fabricated into ampules without special technique. They also reported that the colorless glass was found to meet Type 1, U. S. P. Standards.

Several antibiotics were irradiated and checked for potency on Michigan Memorial Phoenix Project 41. In this study the antibiotics were irradiated in the dry state for twenty-four hours (1.92 million rep in air). Following irradiation, the samples (along with control samples) were dissolved in sterile distilled water in appropriate concentrations and cylinder assay tests were carried out for potency of the various antibiotics, using *Staphylococcus aureus* as the test organism.

Tests for potency of the treated as well as the untreated controls were carried out three days after irradiation. Additional tests for potencies were made on the same solutions six days and again ninety days after irradiation following storage in a refrigerator (4–10°). The results of these tests are presented in Table II.

TABLE II.—THE EFFECT OF GAMMA RADIATION (COBALT-60) ON THE POTENCY OF SEVERAL ANTIBIOTICS

	Potencies—Days After Irradiation (Same Solutions)					
	3 Days		6 Days		90 Days	
Antibiotic[a]	Irradiated, Mm.	Unirradiated, Mm.	Irradiated, Mm.	Unirradiated, Mm.	Irradiated, Mm.	Unirradiated, Mm.
K-Penicillin "G"	37[b]	37	30	32	37	37
Streptomycin HCl	24	24	18	18	13	13
Aureomycin	31	31	25	20	23	24
Chloromycetin[c]	23	23	14	21	16	16
Terramycin	29	24	17	20	21	21

[a] Physical appearance of the antibiotic powders, or crystals, immediately after removal from the cobalt-60 vault following twenty-four hours exposure to the gamma rays:
Penicillin: No color change; no apparent change in solubility in water.
Streptomycin: Slight change in color of treated powder to gray.
Aureomycin: No change in color, nor apparent change in solubility.
[b] Figures represent zones of inhibition against *S. aureus* in the cylinder assay test of solutions prepared from treated and untreated powders.
[c] Chloromycetin: (Trademark registered by Parke, Davis & Company for chloramphenicol.) Slight change in color of treated crystals to gray.
Terramycin: No change in color; no apparent change in solubility.

In summarizing the data given in Table II, it may be noted that in general and within the limits of experimental error of these preliminary tests, there appear to be no appreciable over-all differences in potency between the irradiated and unirradiated samples of the several antibiotics. With the exception of penicillin, which appeared to retain its potency while in solution for ninety days (both in the irradiated and unirradiated series), the solutions showed losses in potencies which appeared comparable in degree in the irradiated and the unirradiated control solutions. The footnotes under the table indicate the physical appearances of the powders or crystals soon after irradiation. A slight change in color, from white to gray, was noted in the irradiated samples of streptomycin and Chloromycetin. However, there did not appear to be any differences in the solubilities of these samples as compared to the unirradiated ones.

CALIBRATION OF GAMMA SOURCE

In order that other workers in this field may have a basis for comparison of results, it is essential that dosages be reported as accurately as possible. This requires an accurate calibration. The latter is not a simple matter when using a large gamma flux because instruments are not readily available which will accurately measure such a high flux. A calculation (4) based on the geometry of the source predicted a dose rate of 78,300 rep per hour in air which is in general agreement with the four different methods of calibration.

The ferrous sulfate method of Weiss and others (5) was used in a preliminary calibration. The data gave a dose rate of 62,300 rep per hour (in water), using a value of 15.4 micromoles per liter per 1,000 rep as recommended by Weiss. However, this calibration was not considered completely satisfactory and is being checked by additional measurements.

A chloroform-water mixture was hydrolyzed by gamma radiation to produce hydrochloric acid and the latter used for calibration (6, 7). Hydrolysis by known dose rates (in air) was determined by *p*H measurements and compared with measurements made within the source. A value of 74,000 rep per hour (in air) was obtained for the dose rate by this method.

Dupont Adlux film and Victoreen r-meter chambers were compared with ionization chambers of the "Cutie pie" type calibrated for gamma radiation. Readings were taken at a point of suitable dose rate outside the lead container and about one foot above the center of the source. The dose rate in this position was within the limited range of the Cutie pie meters. Also at this position and dose rate, the Cutie pie meters and r-meters yielded essentially identical results. The Adlux films were calibrated at this position. Only the films and r-meters could be used to measure the dose rates within the source because the flux was so large. Both the film measurement and the r-meter gave similar values with a mean of 79,200 ± 9,000 rep per hour (in air).

Based on these measurements and calculations, a value of 79,000 rep per hour (in air) as of July 1, 1952, is used for the dose rate of the University of Michigan kilocurie source. A calculation (4) shows that when the source was received in June of 1951, the dose in air was about 90,000 rep per hour.

The dose rate in water solutions or tissue is of greater interest than the dose rate in air, but is less well known. On the basis of the calibration with ferrous sulfate, the dose rate in water appears to be somewhat smaller than the value in air. Other workers (8) suggest that the value of gamma dose rate in tissue would be about 0.95 of the dose rate in air.

SUMMARY

1. The sterilizing ability of gamma radiation was investigated by subjecting both uncontaminated and contaminated samples of a variety of pharmaceuticals in finished package form to the radiation from a one-kilocurie cobalt-60 source.

2. All of the contaminated samples were rendered sterile after twenty-four hours of irradiation.

3. All of the pharmaceuticals except one, a pituitary substance extract, showed no significant deterioration from the irradiation.

4. The ordinary glass containers used in packaging the pharmaceuticals suffered marked discoloration when subjected to irradiation levels necessary to achieve sterilization.

5. Except for glass discoloration and the possible deterioration of a limited number of products such as pituitary substances, the sterilization of pharmaceuticals in finished package form via gamma radiation is considered to be a practical and valuable addition to the various sterilization methods used by the pharmaceutical industry.

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A Pharmacognostical Study of Rauwolfia*

By HEBER W. YOUNGKEN, Sr.†

The history of the drug Rauwolfia Serpentina and of *Rauwolfia canescens* is reviewed. A description is given of *Rauwolfia serpentina*, *R. canescens*, *R. micrantha*, and *R. densiflora* plants and their distribution. The histology of the roots of these species and of *R. perakensis* is described.

THIS STUDY was undertaken after finding the available literature on *Rauwolfia* to be inadequate in determining the identity of many segments of roots in commercial lots offered on the American market as *Rauwolfia Serpentina*.[1] Huge amounts of *Rauwolfia* roots have entered the United States within the past two years to be processed by pharmaceutical manufacturers into products for use in the treatment of hypertension. These products at the present consist of tablets of the powdered whole drug or tablets containing various of the alkaloids of *Rauwolfia*. There has been a growing demand in pharmaceutical industry and among physicians for information on *Rauwolfia*, its physical appearance, constituents, and action, as well as for means of distinguishing *Rauwolfia serpentina* from its adulterants and substitutes. While our present knowledge on these subjects is incomplete, considerable is known. It is the purpose of this paper to assemble the more important developments in the history of this drug and to report the author's findings on its pharmacognosy.

The genus *Rauwolfia* L. of the *Apocynaceæ* comprises about 131 valid species (1) which are distributed in the tropical regions of both hemispheres. Eight of these have been reported to occur in India (1). The roots and leaves of at least two *Rauwolfia* species, namely, *Rauwolfia serpentina* Benth. ex Kurz and *R. canescens* L. have long been used in Ayurvedic medicine as remedies for various ailments. Those of *Rauwolfia serpentina* are mentioned in the Charak (1000 to 800 B.C.), one of the old books of Hindu medicine, as an antidote against the bites of snakes and the stings of insects, but that use has not been supported scientifically. The rhizome and roots have been more recently employed in the modern medicine of India as a hypnotic and sedative in insomnia and insanity, and in the treatment of hypertension.

Under the title of Rauwolfia, the root is recognized in the *Indian Pharmacopoeial List* of 1946 as "The dried roots of *Rauwolfia serpentina* Benth. (Fam. *Apocynaceæ*) with the bark intact, collected in autumn from 3 to 4 year plants, and containing not more than 2 per cent of other organic matter and not less than 0.8 per cent of total alkaloids of Rauwolfia." This work briefly describes it, gives its dose as from 1 to 2 Gm., and its preparations as liquid extract, dried extract, and tincture (2).

Rauwolfia serpentina has recently been introduced into clinical medicine in the United States as the result of chemical and pharmacological investigations carried out mainly in India and later in this country, which showed it to possess therapeutic properties of promise in the treatment of some cases of hypertension.

Between 1931 and 1939 S. Siddiqui and R. H. Siddiqui (3) carried out extensive chemical studies on the roots of *Rauwolfia serpentina* growing in Bihar and isolated two groups of alkaloids. The first of these was called the ajmaline group and consisted of ajmaline ($C_{20}H_{26}O_2N_2$, m. p. 158–160°), ajmalinine ($C_{20}H_{22}O_4N$, m. p. 180–185°), and ajmalicine (decomp. 250–252°) which represent white weak bases; the second known as the serpentine group consisted of serpentine ($C_{21}H_{22}O_4N$, m. p. 153–154°) and serpentinine (decomp. 263–265°) which represent yellow, strong crystalline bases. These findings were confirmed by van Itallie and Steenhauer (4). These workers found that the alkaloids of the ajmaline group, when tested on frogs, acted as depressants to the heart, nerves, and respiration and that those of the serpentine group depressed the nerves and respiration and stimulated the heart.

Siddiqui (3), in 1939, reported that the roots of the Dehra Dun variety of *R. serpentina* differed in their alkaloidal contents from the roots of the same species growing in Bihar, as the former contained very little of the yellow bases, while the white bases differed from the ajmaline group in their chemical composition and yield. Two alkaloids from the Dehra Dun variety were isolated by Siddiqui and provisionally named iso-ajmaline (m. p. 265–266°) and neo-ajmaline (m. p. 205–

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[1] The author is indebted to Mr. F. J. Hailer and Dr. G. Maison of Riker Laboratories, and Drs. S. Rajagopalan, S. Prasad, and S. C. Datta, who generously donated authentic specimens; and to Riker Laboratories, S. B. Penick Co., Columbus Pharmacal Co., and The Prentiss Drug and Chemical Co., who supplied samples of commercial lots of Rauwolfia roots.

207°). Both of these alkaloids were found to have a slight stimulant action on the nervous system followed by marked depression. Both were depressant to the muscles of the heart, blood vessels, and intestines. Both lowered blood pressure in the intact spinal and decerebrate animals. Neo-ajmaline was found to exert a powerful stimulant action and iso-ajmaline, a mild depressant action on the uterus of the cat and guinea pig.

In 1941, Mookerjee (5) isolated an alkaloid called rauwolscine ($C_{21}H_{26}O_3N_2$) from *Rauwolfia canescens* L., finding 0.1 per cent in the root bark, 0.2 per cent in the stem bark, and 0.5 per cent in the leaves. This alkaloid differed from the alkaloids in *R. serpentina* in chemical properties, and resembled yohimbine. Its action, also, was found to differ by Chakravarti (6).

In 1942, Chopra and associates (7) reported the results of a comparative study of the pharmacological action of the alkaloids of *R. serpentina* and their role in experimental hypertension. In 1946, Gupta, Dutta, Ghosh, and Kahali (8) found oleoresins in the root drug of *Rauwolfia serpentina* and reported it to possess specific sedative and hypnotic action. The same workers also reported that the drug contained about 1.21 to 1.36 per cent of total alkaloids.

Other constituents occurring in this drug are sterols, an oxymethylanthraquinone derivative, fumaric acid, oleic acid, glucose, sucrose, unsaturated alcohols, calcium oxalate, and a fluorescent substance.

In 1953 Nelson and Schlagel (9) reported that the potency of *Rauwolfia serpentina* was one-tenth that of *Veratrum viride* in terms of the crude drug, that the hypotensive action resides in the alkaloidal fraction and that serpentine was more potent than the ajmaline alkaloids.

Most of the past investigations of this plant have been chemical and pharmacological, while relatively few were concerned with its pharmacognosy. In 1885, Dymock (10) briefly described it and reported the presence of an alkaloid, resin, and volatile oil. Several writers on Indian Materia Medica, including Chopra (11), Nadkarni (12), Khori and Katrak (13) who followed Dymock, added little of pharmacognostical significance to Dymock's report. The most important past contributions in this field have been those of Datta (14), Wallis and Rohatgi (15), and Rao (16). In 1949, Datta (14) reported on the morphological and some of the histological features of roots of some commercial varieties of *Rauwolfia serpentina* and *R. canescens* and their powders. He found the range of starch grain index useful in distinguishing between varieties of *Rauwolfia serpentina* and between the roots of that species and *R. canescens*. Wallis and Rohatgi (15) limited their report of the same year to the pharmacognosy of *Rauwolfia serpentina* rhizomes and roots, stressing the microscopical features of those organs and the powdered drug.

In 1950, Rao (16) reported the results of his studies on the roots, stems, and leaves of *Rauwolfia serpentina* and *R. canescens*, and was the first to distinguish the principal diagnostical differences between the leaves of the two species. He stated that the roots of *R. serpentina* and *R. canescens* were sold indiscriminately in the drug market under the same vernacular name, "Chota Chand."

However, the problem of determining adulteration, today, is found to be more difficult since commercial lots labeled "Rauwolfia Serpentina" examined by the writer occasionally consist of or contain roots of species other than *R. canescens*. Moreover, the stratified cork and starch reported diagnostic for *R. serpentina* roots and the presence of stone cells in the phloem of *R. canescens* occurred in roots of other species examined by the writer. In a very large number of commercial samples examined by him, he found that, while the roots of *R. canescens* have been the most frequent of the adulterants and substitutes detected, the roots of *Rauwolfia densiflora*, *R. micrantha*, and *R. perakensis* were also used as substitutes. All of these species occur in India, and some of them elsewhere in Southern Asia, and *R. canescens* is reported to occur frequently in some areas among *R. serpentina* plants (16). Recently, the author found two commercial lots to consist of roots of another species of *Rauwolfia* whose source remains undetermined.

MATERIALS

The materials used in this study included the following: (*a*) Two representative samples of about a pound each of the roots and rhizomes of *Rauwolfia serpentina* and *R. canescens* from S. C. Datta of the Pharmacognosy Laboratory, Government of India, Calcutta.

(*b*) Samples of botanically authenticated roots of *Rauwolfia serpentina*, *R. canescens*, *R. densiflora*, *R. perakensis*, and *R. micrantha*, obtained from Dr. S. Rajagopalan of Madras, India.

(*c*) Herbarium sheets of *Rauwolfia serpentina*, *R. canescens*, *R. densiflora*, and *R. micrantha* obtained from Dr. S. Prasad of Benares Hindu University, Benares, India.

(*d*) Herbarium specimens of *Rauwolfia perakensis* from the Gray Herbarium, Cambridge, Mass., and herbarium sheets of *Rauwolfia serpentina* and *R. canescens* from the Arnold Arboretum.

(*e*) A very large number of samples of commercial lots labeled "Rauwolfia Serpentina Roots" supplied by Riker Laboratories, Inc., and several crude drug houses.

(*f*) Sections of macerated material, and powders of representative segments of all of the aforementioned roots and rhizomes.

RAUWOLFIA SERPENTINA

Plant.—*Rauwolfia serpentina* Benth. ex Kurz (17) is an erect, glabrous shrub (Fig. 1) up to about 1 m. in height with cylindrical stems having pale bark, and emitting a light colored, viscous latex when broken. Its leaves are simple, opposite, or more usually in whorls of 3 to 5, when mature from 7 to 18 cm. long, and from 2.5 to 5 cm. broad, obovate, lanceolate to oblong-lanceolate, with entire margin, acute to acuminate apex and unequal base, tapering into a short petiole. Its flowers are white to pale rose (in corolla tube) and are arranged in terminal and axillary cymes. The fruit is a single 2-lobed drupe, purplish black at maturity. It is native to India, Burma, Ceylon, Cochin China, Malaya, Java, and the Philippines. According to a note received from R. L. Badhwar, of the Forest Research Institute of Dehra Dun, it occurs in moist and hot regions of the sub-Himalayan region from the Punjab eastward to the Khasi Hills in Assam, especially in the Siwaliks, Rohilkhand, Oudh, and Gorakhpur, ascending to an altitude of 4,000 ft., also in Konkan, N. Kanara, S. Mahratta, the eastern and western Ghats of Madras State up to 3,000 ft., in many districts of Bihar, such as Patna and Bhagalpur, in north and central Bengal, and in the Andamans.

Description of Roots and Rhizomes.—The physical characteristics of *R. serpentina* or Sarpagandha roots are, in general, as follows: The entire root (Fig. 2) is conical, tortuous, and curved, bearing a number of curved, secondary rootlets which are more numerous in the upper portion where they become woody and rigid; up to about 22 mm. in width at the summit; externally grayish yellow, light brown or brown, slightly wrinkled to rough, and irregularly longitudinally wrinkled or ridged in thicker and older parts. The commercial drug occurs in cylindrical to cylindrical-tapering segments up to about 10 cm. in length and from about 3–22 mm. in diameter, some of the segments bearing the woody crown (rhizome) with attached stem bases. Its fracture is short and irregular, breaking in sufficiently long segments with a snap and often exhibiting some projecting strands of periderm along the margin. The freshly fractured surface exhibits a grayish-yellow bark which readily peels from the wood, and a pale yellow wood, the latter hard, and up to about four-fifths the diameter of the root. The odor is indistinct, suggesting potatoes, the taste very bitter.

Fig. 1.—*Rauwolfia serpentina* Benth. ex Kurz.

Fig. 2.—*Rauwolfia serpentina* Benth. ex Kurz. Root of Dehra Dun variety.

Histology.—*Rauwolfia serpentina* root exhibits the typical general structure of a perennial dicotyl root of secondary growth. Its special characteristics are as follows: the cork shows stratification and is composed of from 2 to 8 alternating strips or zones of smaller and radially narrower and larger and radially broader cells (Fig. 3*K*). The smaller and narrower cork cells consist of 2 to 5 layers of cells with suberized walls. The larger cork cells occur in tangential zones of 1 to 5 layers, occasionally more, between two bands of smaller cork cells. The cells in the middle of the larger cork zones are larger than those bordering the zones of smaller cells, and are up to about 90 μ in radial length and up to 75 μ in tangential width. They have thin, lignified walls. The

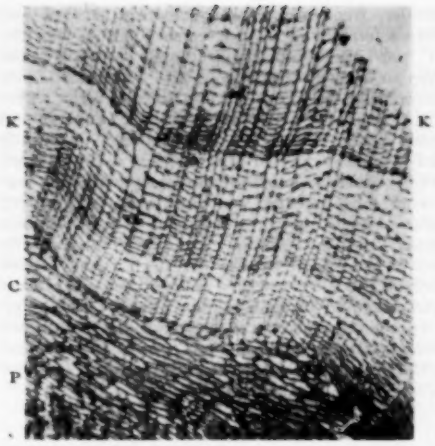

Fig. 3.—*Rauwolfia serpentina*. Root. Transverse section of outer region of bark showing stratified cork (*K*), secondary cortex (*C*), and outer portion of phloem (*P*) of a segment 15 mm. in thickness. Photomicrograph × 20.

smaller cork cells were up to 75 μ in tangential width. In longitudinal sections the cork cells were polygonal in outline and measured up to 90 μ x 70 μ.

The secondary cortex occupied a zone of up to about 860 μ in width and is composed of tangentially elongated to isodiametric parenchyma cells which contain starch. Short latex cells, containing a brown resin, occurred in short rows and singly in this region.

The secondary phloem (Fig. 4*P*) consists of starch- and crystal-bearing phloem parenchyma, and sieve tissue, traversed by phloem rays which curve outward. Some of the outer cells and rays contain a brown resin while other phloem cells contain starch grains up to about 14 μ and a lesser number tabular to angular crystals of calcium oxalate from 3–20 μ in length.

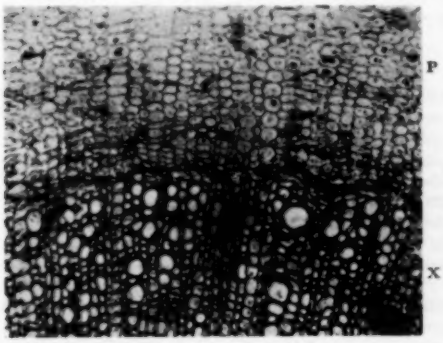

Fig. 4.—*Rauwolfia serpentina*. Root. Transverse section of inner region of bark and outer region of wood, showing secondary phloem (*P*), and secondary xylem (*X*). The dark wavy line shown between these regions is the cambium. Photomicrograph × 40.

The secondary xylem (Fig. 4X) occupies about four-fifths the diameter of the root. It is composed of numerous wood wedges separated by xylem rays. The wedges contain vessels, tracheids, wood parenchyma cells, wood fibers, and shorter rays—all of which possess lignified walls. The vessels were usually fewer in number and on the average smaller in size than those of the wood wedges of *Rauwolfia canescens*, *R. densiflora*, and *R. perakensis*. They were up to 360 μ in length and up to 48 μ, rarely up to 57 μ in width. The vessel elements exhibit mainly simple pits but those adjacent to the xylem rays possess bordered pits. They are cylindrical in shape with mostly oblique to transverse end walls, marked by openings surrounded by prominent perforation rims, but a number of them possess abruptly tapered and closed end walls with the perforation plates on the side walls. Some of the vessels showed tyloses and some contained gummy lignin in their cavities. Tracheids, wood fibers, and wood parenchyma accompany the vessels. The tracheids are of the pitted type with moderately thick beaded walls and broader lumina. The wood parenchyma cells possess moderately thick pitted walls. In cross section they are polygonal, in longitudinal section, oblong. They are loaded with starch. The wood fibers possess thick, greatly lignified walls with transverse and oblique pits and pointed to bifurcate ends. They range from 200 μ to about 750 μ in length. In transverse sections of the root, they are arranged in tangential bands and radial rows. The xylem rays usually range from 1 to 5 cells in width, but in the roots from the Trichur district (N. Cochin) they were from 1 to 8 cells in width, and those from the Ernakulam district (S. Cochin) were from 1 to 6 cells in width. They contained starch.

The powdered root as well as scrapings, when examined in water mounts, exhibited numerous starch grains, simple and 2- to 3-compound, the individual grains being spheroidal, ovate, muller-shaped, and plano- to angular-convex with a Y-shaped, stellate, or irregularly cleft hilum. The unaltered grains were up to 27 μ, rarely 34 μ in diameter, the altered grains up to 50 μ in diameter. The larger unaltered ones showed distinct polarization crosses.

Rauwolfia serpentina rhizome in cross and longitudinal sections exhibits a cork somewhat similar to that of the root, a phellogen of rectangular cells, a phelloderm of up to about 6 layers of parenchyma which contain scattered latex cells with resinous contents, a narrow pericycle which contains pericyclic fibers arranged singly or in small groups of 2 to 5, a broad circle of bicollateral vascular bundles separated by vascular rays, and a small central pith. The parenchymatous cells of cortex, pericycle, xylem, phloem, and pith contain starch grains similar to those described in the root.

The pericyclic fibers are typically apocynaceous in character, showing thick, nonlignified walls, and tapering, often lobed ends, and with subterminal enlargements having thin walls and broad lumen.

The wood wedges of the xylem are composed chiefly of starch-bearing parenchyma, through which course radially arranged pitted vessels, tracheids, and fibers. A number of tyloses and masses of gummy lignin occur in many of the vessels. The vessel elements measured up to 485 μ in length and up to 45 μ in width. The wood fibers were generally

less wavy than those of the root and measured up to 750 μ in length and up to 32 μ in diameter. The xylem rays were from 1 to 4 cells in width. Their walls were lignified and pitted. Beneath the xylem and embedded in the outer region of the pith are a variable number of internal phloem strands.

The pith is composed of starch parenchyma and scattered short latex cells with yellowish contents, staining brown with iodine T. S.

RAUWOLFIA CANESCENS

Rauwolfia canescens L. (Fig. 5) is a spreading, dichotomously branched shrub with pubescent branches that attain a height of 2 m. Its leaves are borne in whorls of 3, are elliptic lanceolate in outline, from 4 to 10 cm. in length, and 1.5 to 6 cm. in breadth. The flowers are small, white, pentamerous, with an urceolate calyx, and tubular pubescent corolla with rounded lobes.

The fruit is a red globular drupe containing two 1-seeded putamens. It is native to India and Burma, occurring in many areas where *R. serpentina* abounds. It also grows in the West Indies. The root of this plant has long been used in the popular medicine of India interchangeably with that of *R. serpentina*. It has been the most frequent substitute and adulterant for the latter in the numerous lots purported to be Rauwolfia Serpentina Root, examined by the author.

Physical Characteristics.—The entire root of *Rauwolfia canescens* examined from a herbarium sheet obtained from S. Prasad was conical, curved, and tortuous, nearly 3 ft. in length, with a long, slender, frequently curved, tapering process, and bore a number of slender, curved, fibrous rootlets. It was irregularly longitudinally wrinkled and grayish brown externally. The commercial root occurs in mostly curved, sometimes straight, cylindrical segments, up to about 10 cm. in length and from 4 mm. to 2.5 cm. in diameter; externally grayish brown, brown or dark gray, reddish brown where abraded, longitudinally wrinkled, some segments show circular scars and stubs of rootlets, others bear the crown with 2 or more attached stem bases; the basal portion of the latter occasionally exhibits small cork plates; the bark is readily peeled from the wood; fracture of thin pieces are short and irregular, of thick pieces, irregular and splintery, the smoothed cut surface showing a light brown bark and a pale yellow wood which occupies four-fifths to nine-tenths the width of the root; the odor is indistinct; the taste is very bitter.

Fig. 5.—*Rauwolfia canescens* L. × 1/3.

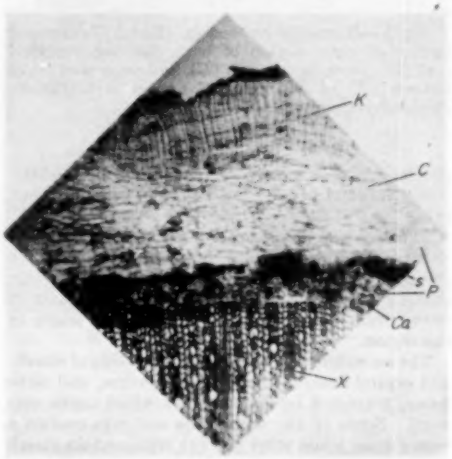

Fig. 6.—*Rauwolfia canescens* L. Transverse section of representative portion of the root, showing cork (*K*), secondary cortex (*C*), phloem (*P*) containing sclerenchyma (*s*), cambium (*Ca*), and outer portion of the secondary xylem (*X*). Photomicrograph × 20.

Histology of the Root.—In transverse section (Fig. 6) the cork exhibits up to about 45 layers of rectangular cells arranged in radial rows with interspersed radial rows of isodiametric cells. The cells possess suberized and lignified walls and are up to about 65 μ in tangential length and from 6 μ to about 20 μ in radial width. In surface section the cork cells were polygonal and measured from 20 to 65 μ in diameter. The cork is not stratified as in *R. serpentina*.

The phellogen consists of 1 or 2 layers of thin, rectangular and square cells. The secondary cortex is composed of rounded to tangentially elongated parenchyma, its cells containing starch grains or rhombohedral to cubical crystals of calcium oxalate, the latter from 8 μ to about 35 μ in diameter or length.

The secondary phloem occupies a band of up to 25 or more layers consisting of starch- or crystal-bearing phloem parenchyma, sieve cells and tubes, com-

panion cells, phloem rays, and scattered sclerenchyma elements (*s*). The crystal cells contained calcium oxalate crystals of varying form and size from tiny prisms to larger tabular forms, some of the latter attaining a length of 38 μ. The starch-bearing phloem rays pursued a curved course and consisted of mostly tangentially elongated, thin-walled cells. In tangential-longitudinal sections, they were from 1 to 5 cells in width. The sclerenchyma elements were distributed not only in the inner part of the phloem as reported by Rao (16) but in middle and outer parts as well. They included stone cells of many forms and varying thicknesses of their walls and lumina, long rod-like forms intermediate between stone cells and fibers, and sclerenchyma fibers, the latter up to 800 μ in length. They were isolated and in groups of from 2 to 12. A very diagnostic form occurred in the tissue prepared by Schulze's maceration method, being irregularly lobed with pointed projections and with an interrupted lumen. A number of latex cells with a brown resin occur in the outer phloem region.

The cambium is composed of a narrow line of thin-walled cells.

The secondary xylem consists of numerous radiately elongated wood wedges separated by usually narrower xylem rays. The wood wedges showed more numerous vessels than those of the root of *R. serpentina* of similar diameter. They are cylindrical with simple pitted to bordered pitted walls. While many with transverse and oblique end walls and end wall perforations occur, the majority of those studied showed tapered end walls with side-wall openings. They ranged from about 180 μ to 655 μ in length and from 28 μ to 70 μ in width.

The tracheids possess tapered ends and simple pits. They were up to about 1,000 μ in length and up to 30 μ in width.

The wood parenchyma, like the other xylem elements, possesses lignified walls. Its walls have simple pits. The xylem rays, in tangential-longitudinal sections, were from 1 to 5 cells in width. They possessed simple to semi-bordered pits in their walls and their cavities contained starch grains.

The starch grains are of the same types as in the root of *R. serpentina* but appeared less abundant. Most of them were under 15 μ but occasional grains measured up to 26 μ in diameter.

The wood fibers varied in length from 400 μ to 1500 μ and were up to 30 μ in width. They possess simple and oblique pits in their greatly lignified walls. A number of them show a series of irregular curvatures or projections above one of the ends.

Histology of the Rhizome.—The cork of the rhizome shows a similar stratification to that of the root. The cortex is differentiated into an outer zone of collenchyma and an inner zone of starch-bearing parenchyma in which are scattered latex cells. The pericycle consists of a narrow band of starch parenchyma in which are found groups of pericyclic fibers, the latter up to about 1200 μ in length and up to about 45 μ in width. They possess long attenuated ends and bulb-like dilatations.

The bundles are of the bicollateral type, constituted of external phloem, xylem, and internal phloem. The phloem parenchyma contains starch and calcium oxalate prisms. The xylem is broad and possesses radially arranged wood wedges separated by xylem rays. The xylem vessels are accompanied by tracheids and wood fibers and xylem cells. The vessels are arranged singly and in radial rows. Their walls are pitted, those adjacent to the rays possessing bordered pits. Most of them have tapered ends and side-wall openings; fewer have terminal openings. Some of the vessels possess tyloses and contain gummy lignin. The tracheids possess simple pits and tapered ends. The wood fibers measure up to 1,120 μ in length and up to about 25 μ in width. The wood parenchyma cells are pitted and contain starch. The xylem rays are from 1 to 2 cells in width. Their walls are lignified and pitted. The internal phloem consists of a narrow band of phloem cells, sieve tubes, and companion cells traversed by narrow phloem rays.

The pith in the center of the rhizome consists of isodiametric parenchyma whose cells contain starch and calcium oxalate, and scattered stone cells which are isolated and in groups. Scattered among the parenchyma cells are latex cells with resin content.

Fig. 7.—*Rauwolfia micrantha* Hook. fil. Leaf and fruiting branch (*A*); segment of root (*B*). × 1/3.

RAUWOLFIA MICRANTHA

Rauwolfia micrantha Hooker f. (Fig. 7) is a shrub native to Malabar with slender branches that bear opposite to 3-whorled leaves with elliptic-lanceolate and oblanceolate, acuminate blades, few flowered cymes of small tubular flowers, and ovate drupe fruits. A number of commercial samples of Rauwolfia Root gathered in the Coastal Plain area of India and offered as "Rauwolfia Serpentina" were determined by the author to represent this species.

Physical Characteristics of the Root.—The *R. micrantha* root occurs in straight, curved, or tortuous segments up to about 10 cm. in length and from 2 to 2.5 cm. in diameter; externally yellowish brown, the bark nearly smooth to usually longitudinally wrinkled, often scaly, and easily peeled from wood; the fracture of thinner pieces short and irregular, of thicker pieces, irregular and splintery; wood pale yellow and very hard in texture; the odor is indistinct; the taste is very bitter.

Histology.—The cork shows stratification (Fig. 8*A*, *k*) and consists of 2 to 7 or more bands of alternating larger and radiately broader cells with lignified walls and smaller and radiately narrower cells. The larger cork cells in cross sections are up to 75 μ in tangential length and up to 48 μ in radial width. The smaller cork cells occur in 1 to 4 layered bands, the larger cork cells in 2 to 9 layered bands. The secondary cortex consists of starch- and crystal-bearing parenchyma comprising up to about 14 layers of tangentially elongated cells. The subjacent secondary phloem consists of a band of starch- and crystal-bearing phloem parenchyma, sieve cells and tubes, and companion cells, traversed by phloem rays, from 1 to 4 cells in width, which curve outward. Numerous monoclinic prisms, smaller angular crystals, and a number of twin crystals of calcium oxalate mostly up to 20 μ, occasionally up to 38 μ in length, occur in the phloem parenchyma. Scattered through the phloem are numerous stone cells, fiber-like stone cells, and fibers. They occur mostly singly but also in groups. The fiber-like stone cells were up to 361 μ in length, up to 38 μ in width and possessed beaded walls. The fibers measured up to 800 μ in length and up to 38 μ in width.

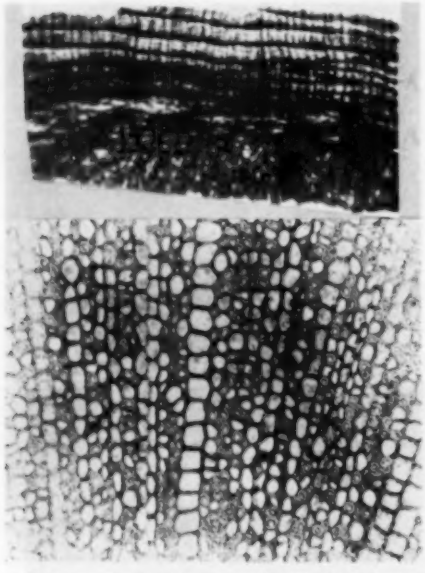

Fig. 8.—*Rauwolfia micrantha* Hook. fil. Root. (*A*)—Transverse section of bark showing stratified cork *k*; (*B*)—transverse section of the secondary xylem region of the wood. Photomicrograph × 40.

The xylem consists of a broad zone of numerous wood wedges separated by wood rays from 1 to 5 cells in width and the walls of all of its elements are lignified. The wood wedges contain numerous tracheids and wood fibers with relatively fewer vessels, the latter arranged singly in radiate groups, in pairs and in nests of up to 7, as viewed in cross sections. The vessel elements have pitted and bordered pitted walls, are circular, oval, or polygonal in cross section, and have transverse, oblique, and tapered end walls. Openings occur in both end and side walls in different vessels. The vessel elements measured up to 450 μ in length and up to 76 μ in diameter. A number showed tyloses. The wood parenchyma cells were rectangular in longitudinal section with pitted walls, starch contents, and up to 133 μ in length. The wood fibers were up to 974 μ in length and 38 μ in width. They possessed tapered ends, walls with simple and oblique pits up to 11 μ in thickness, and strongly lignified. The tracheids possessed simple to bordered pits and attained a length of 600 μ.

Fig. 9.—*Rauwolfia densiflora* Benth. ex Hook. fil. (*A*)—Leafy twig, to right, portions of cyme bearing fruits. (*B*)—Segment of root. × 1/6.

RAUWOLFIA DENSIFLORA

Rauwolfia densiflora Benth. (Fig. 9) is a shrub native to the Khasia Mountains of India and to Ceylon. It occurs in the Western Ghats from Concan southward. It is characterized by its obovate to oblanceolate, acuminate leaves in whorls of 3 to 4, cymes of rose-red to white tubular flowers whose sepals are about half the length of the corolla tube, and by its ellipsoidal drupe fruits, the latter up to 12 mm. long in the materials examined. The authentic root specimens studied and described below were from the Mahableswar collection.

Physical Characteristics of R. densiflora Roots.—The dried roots occur in curved and tortuous segments up to 9 cm. in length and from 2 to 25 mm. in

width; externally brown to yellowish brown; irregularly longitudinally fissured and ridged; the fracture of the thin pieces short, of the thick pieces, short in bark, long splintery in wood; wood pale yellow and very dense in texture; the odor is indistinct; the taste is bitter.

Histology.—The cork is stratified and consists of up to about 12 alternating bands of larger and radially broader and smaller and radially narrower cells with suberized and lignified walls. The larger cork cells were up to 76 μ in radial length and up to 68 μ in tangential width in cross sections. The phellogen consists of 1 to 2 layers of thin-walled rectangular and square cells. The secondary cortex occupies a narrow band of up to about 10 layers of tangentially elongated starch- and crystal-bearing parenchyma cells among which are scattered stone cells arranged singly and in small groups. The secondary phloem is characterized by parenchyma cells similar to the cortex in which are imbedded a large number of isodiametric and fiber-like stone cells. The smaller stone cells varied greatly in outline and size. They are up to about 169 μ in length and up to about 80 μ in width. The fiber-like stone cells were more or less rod-like with rounded, obtuse or truncate ends and up to about 640 μ in length and 80 μ in width. The walls of both types were usually thicker than the lumen.

The xylem (Fig. 10) consisted of numerous wood wedges separated by starch-bearing xylem rays from 1 to 6 cells in width. The wood wedges contained numerous vessels, accompanied by tracheids and wood parenchyma. The vessel elements are cylindrical with pitted to bordered pitted walls and with oblique, circular, to tapered end walls with openings either at the ends or, in all of the taper-ended vessels, on the side walls. They were up to about 480 μ in length and up to about 80 μ in width and more numerous than in sections of the other *Rauwolfia* roots studied. In cross sections of the root, they are elliptic, oval, or circular in outline, and their arrangement is very diagnostic for *R. densiflora*, most of them forming concentric circles in the wood (Fig. 10). The wood fibers appeared to be longer in maximum length than those of the other species here reported. They were up to 2,100 μ in length and up to 34 μ in width. Their walls averaged up to about 10 μ in thickness and showed oblique pits. They were undulate in outline and showed attenuated, obtusely pointed ends. All of the xylem elements possess lignified walls.

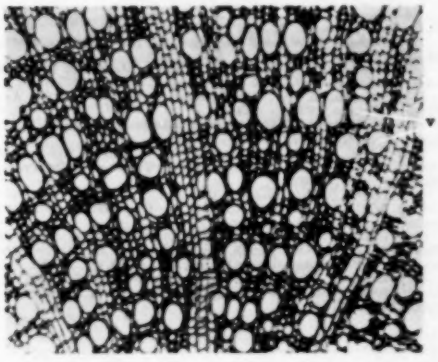

Fig. 10.—*Rauwolfia densiflora*. Photomicrograph (× 40) of a portion of the secondary xylem of the root, as viewed in cross section, showing the characteristic arrangement of the vessels (*v*) in concentric circles.

Starch grains or crystals occur in the parenchymatous cells of phloem, cortex, and wood. The starch grains are simple and 2- to 4-compound, the unaltered grains being up to 20 μ and the altered grains up to about 40 μ in diameter. The calcium oxalate crystals occur in rhombohedral monoclinic prisms and twin prisms and are up to 38 μ in length. Many of the smaller prisms are cuboidal in outline, and twin prisms are numerous.

RAUWOLFIA PERAKENSIS

The roots of *Rauwolfia perakensis* King and Gamble here described were of Malayan origin and from the authentic collection of S. Rajagopalan. The species yielding them is a subshrub, native to Southern Asia.

Physical Characteristics.—The entire root is long, conical, and tortuous. Segments of it, as detected rarely in commercial lots offered as *R. serpentina*, are cylindrical, curved, or tortuous, up to 4.5 cm. in length and from 1.5 to 2 cm. in diameter. Externally the thin pieces are light brown, smooth to longitudinally striate; the fracture of these is short with projecting splinters of bark along the margin. Externally, the thicker pieces are grayish brown with pinkish-brown to purplish abraded areas, irregularly longitudinally wrinkled to ridged with occasional pieces showing stiff rootlet stumps; the texture is of bony hardness, unbreakable by hand. Internally, the sawed cross surface shows a bark up to about 0.8 mm. in width and a pale yellow wood up to nine-tenths the diameter of the root. The odor is indistinct; the taste is bitter.

Histology.—(Fig. 11) The cork is stratified and consists of from 1 to 5 wavy bands of smaller and tangentially elongated cork cells alternating with a like number of strips of larger and radiately elongated cork cells with lignified walls. The larger cork cells attained a greater size than those of *R. serpentina* and the other species here reported, up to 171 μ x 57 μ in cross sections. The secondary cortex consists of up to about 6 layers of starch- and crystal-bearing parenchyma in which are interspersed stone cells, most of which were arranged singly in the sections studied. The phloem region contains starch- and crystal-parenchyma, sieve tissue, and scattered stone cells and fibers, the latter up to about 700 μ in length, and is traversed by phloem rays mostly from 1 to 2 cells in width. The secondary xylem shows a radiate arrangement of wood wedges separated by starch and crystal-bearing xylem rays, the latter mostly from 1 to 2 cells, occasionally from 1 to 3 cells in width. The vessel elements exhibit simple pitted and bordered pitted side walls and oblique to circular end walls and openings as well as tapered ends with openings on the side walls. They are mostly up to 38 μ in width, occasionally up to 67 μ. In cross sections the vessels are mostly isolated singly, more rarely occurring in radial and tangential groups of 2 to 5. The wood fibers possess oblique pits in their highly lignified walls. They measured up to 935 μ in length and up to 19 μ in width. All the xylem elements were lignified.

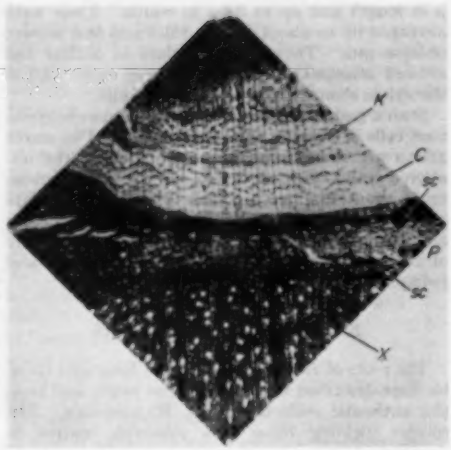

Fig. 11.—*Rauwolfia perakensis* King and Gamble. Photomicrograph of a representative portion of a cross section of the root × 20, showing the stratified cork (*K*), the numerous stone cells and fibers (*sc*) in the secondary cortex (*C*) and phloem (*P*) represented by the dark objects seen in those regions, and the characteristic xylem (*X*) with most of its vessels arranged singly or in short radial groups.

The starch grains are single to 2- to 3-compound, the individual grains spheroidal, ovoid, muller-shaped, and plano- to angular-convex and possess a centric, cleft hilum. They are up to 20 μ in diameter. The crystals of calcium oxalate occurred in monoclinic prisms up to 40 μ and are very numerous in the parenchyma.

SUMMARY

1. The history of the drug *Rauwolfia serpentina* and of *Rauwolfia canescens* is reviewed.

2. A description is given of *Rauwolfia serpentina*, *R. canescens*, *R. micrantha*, and *R. densiflora* plants and their distribution. The first two of these frequently occur in the same areas in India.

3. Of the 8 valid species of *Rauwolfia* occurring in India, botanically authenticated roots of 4 of these, *viz.*, *Rauwolfia serpentina*, *R. canescens*, *R. densiflora*, *R. micrantha*, and *R. perakensis* from Malaya are described as to their physical characteristics and histology.

4. In the numerous samples of commercial lots offered on the American market as "Rauwolfia Serpentina," and examined by the author, roots of *R. canescens* occurred most frequently as substitutes and adulterants, and roots of *R. micrantha*, *R. densiflora*, *R. perakensis*, and of an undetermined species of *Rauwolfia* were occasionally found. Genuine *Rauwolfia serpentina* roots may be distinguished from all of these by the absence of sclerenchyma elements in the secondary phloem and cortex, by the shorter and nonfibrous fracture of its thicker pieces, and by the usually fewer and smaller vessels in the wood wedges.

5. The wood of *R. densiflora* root possesses vessels of greater maximum width than those of the roots of the four other mentioned species.

6. The arrangement of the vessels as seen in cross sections of the root of *R. densiflora* in concentric circles is diagnostic for this species.

7. Latex cells containing a brown resinous secretion occur in the cortex, phloem, and pith of *Rauwolfia serpentina*. They occur singly and in short rows, as seen in longitudinal sections of the root and rhizome. They also occur in *R. canescens*. They were most numerous in the Dehra Dun variety of *R. serpentina*.

8. The cork of the roots of *Rauwolfia serpentina*, *R. micrantha*, *R. densiflora* and *R. perakensis* show stratification in the form of alternating bands of larger and smaller cells, whereas no such stratification was found in the cork of *R. canescens*.

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A New Series of Cholinesterase Inhibitors*

By J. P. LONG and F. W. SCHUELER

In a series of *bis* onium biphenyl derivatives, alteration of substitutions on the quaternary nitrogen produced a wide variation in toxicity and ability to inhibit cholinesterase, as well as other pharmacological responses. Three members of this series were found to be equal to neostigmine bromide as inhibitors of bovine erythrocyte cholinesterase. Toxicity, in all cases, did not parallel cholinesterase inhibiting potency. Mechanism of action and structure-activity relationships are discussed.

ANTICHOLINESTERASE activity has been demonstrated in several classes of compounds (1–3). Studies of synthetic compounds showing high anticholinesterase activity have centered around aromatic carbamates (4), alkyl fluorophosphates (5), alkyl polyphosphates (6), and recently Funke, Depierre, and Krucker (7) have synthesized a series of highly active *bis* neostigmine-type analogs. In the present series (see Table I) anticholinesterase activity varied over a ten thousandfold range with reference to substitution on quaternary nitrogen. Of special interest was the fact that the most toxic member of this series showed the lowest anticholinesterase activity.

The object of this report is to describe in detail the synthesis and pharmacologic tests used in the evaluation of these materials together with a working hypothesis concerning their possible mode of action.

EXPERIMENTAL

Synthetic Procedures.—(*a*) α,α′-di-Pyridyl-4,4′-biacetophenone diiodide (Compound No. 3*a*, Table I) was prepared by heating on the water bath a mixture of 2 Gm. of 4,4′-biacetophenone (8), 4 Gm. of iodine, and 50 cc. of pyridine. After one hour the mixture was poured into 500 cc. of water. The brown precipitate was collected on a filter, suspended in boiling alcohol, recollected on a filter, and washed repeatedly with ether and alcohol (yield 15%).

(*b*) α,α′-di-Pyridyl-4,4′-biacetophenone dichloride (Compound No. 3*b*, Table I) was prepared by heating 2 Gm. of α,α′-dichloro-4,4′-biacetophenone (9) in 15 cc. of pyridine for twenty minutes. The addition of absolute ether precipitated the product which was recrystallized by solution in absolute alcohol followed by precipitation through addition of ether (yield 50%).

(*c*) α,α′-Dibromo-4,4′-biacetophenone (Compound No. 12, Table II) was prepared by dissolving 25 Gm. of biphenyl in 150 cc. of carbon disulfide in a 500-cc. flask. The solution was cooled and 60 Gm. of anhydrous aluminum chloride slowly added with shaking. The flask was next fitted with a reflux condenser and 80 Gm. of bromoacetyl bromide was slowly added through the condenser (ten minutes). There was an immediate liberation of hydrogen bromide and following this initial vigorous reaction, the solution was refluxed until liberation of hydrogen bromide ceased (about two hours). The reaction complex was poured into a mixture of cracked ice, methanol, and hydrochloric acid. The aluminum chloride complex was broken up with stirring and the white product collected on a filter and dried. The product was dissolved in boiling benzene or toluene, filtered, and crystallized by cooling in an ice bath. Two recrystallizations gave a product of constant melting point at a yield of 35%.

(*d*) Compounds 1, 2, and 4 through 11, as shown in Table I, were prepared by dissolving 1.5 Gm. of α,α′-dibromo-4,4′-biacetophenone in 30 cc. of boiling 1,4-dioxane, adding with stirring 5 cc. of the corresponding tertiary amine, and allowing the mixture to stand for thirty minutes. The crystals which formed were collected on a filter, recrystallized by solution in absolute alcohol followed by precipitation through the addition of ether, and finally dried in a vacuum desiccator. Yields ranged from 30 to 50%.

(*e*) α-Pyridyl-4-phenylacetophenone bromide (Compound No. 13, Table II) was prepared by warming 2 Gm. of α-bromo-4-phenylacetophenone with 10 cc. of pyridine, precipitated with absolute ether, and recrystallized as above (yield 50%).

The *bis* onium compounds used in this study are listed in Table I. Table II contains starting materials and mono quaternary compounds. All melting points were determined using a Fisher-Johns melting-point block and are uncorrected.

PHARMACOLOGICAL PROCEDURES

Since the compounds varied widely in anticholinesterase activity, the following three representative compounds were selected from the group for screening on the isolated heart and for the study of their ability to potentiate the acetylcholine muscarinic depressor response: potent cholinesterase inhibitor (Compound No. 3*a*, Table I); intermediate cholinesterase inhibitor (Compound No. 5, Table I); and low in cholinesterase inhibitor potency (Compound No. 11, Table I).

Acute Toxicity.—The approximate LD_{50} were determined in white mice following intraperitoneal injections of the compounds in physiological saline.

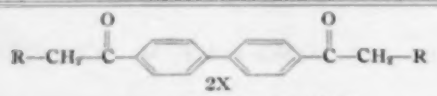
Circulatory and Salivary Experiments.—Dogs were anesthetized with 36 mg./Kg. of pentobarbital

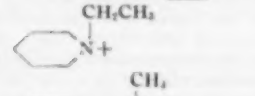
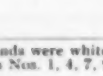
* Received August 27, 1953, from the Department of Pharmacology, College of Medicine, State University of Iowa, Iowa City.

This material is to be submitted to the Graduate College of the State University of Iowa by J. P. Long in partial fulfillment of the requirements for the degree of Doctor of Philosophy.

The work was aided by a research grant from the United States Public Health Service [RG-1262(C4)].

TABLE I.—BIS-PHENACYL AMMONIUM COMPOUNDS



Compound No.[a,b]	R	X	M. P., °C.	Analysis	
				Calcd., %	Found, %
1		Br^-	194–196	Br 27.49	Br 27.70
2		Br^-	>290	Br 27.49	Br 27.40
3a		I^-	>290	N 4.09	N 4.05
3b		Cl^-	>290	Cl 15.21	Cl 15.19
4		Br^-	>290	Br 23.88	Br 23.65
5		Br^-	228–229	Br 25.70	Br 25.00
6		Br^-	216–217	Br 24.24	Br 23.80
7		Br^-	>290	Br 27.49	Br 27.14
8	$(CH_3CH_2)_3N+$	Br^-	>290	Br 26.75	Br 26.90
9		Br^-	222–224	Br 26.75	Br 26.60
10	$(CH_3)_3N+$	Br^-	>290	Br 31.13	Br 31.00
11		Br^-	179–180	Br 27.59	Br 27.20

[a] All compounds were white, crystalline solids except compound No. 3a which was a reddish-brown powder.
[b] Compounds Nos. 1, 4, 7, 9, and 11 were hygroscopic.

sodium. For circulatory experiments the carotid artery was cannulated and recordings were made on a smoked drum using a mercury manometer. All drugs were dissolved in physiological saline and injections were made into the femoral vein. The rate of secretion from the cannulated Wharton's duct was measured by a magnetic recorder.

Cholinesterase Studies.—This portion of study was divided into three parts: (*a*) *in vitro* inhibition of bovine erythrocyte cholinesterase, (*b*) *in vivo* inhibition of dog's cell and plasma cholinesterases, and (*c*) *in vitro* inhibition of dog's cell and plasma cholinesterases. Standard manometric techniques (11) were employed to measure the cholinesterase activity.

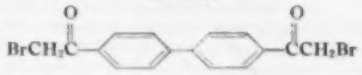
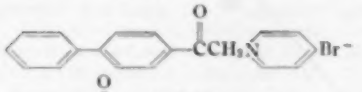
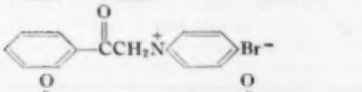
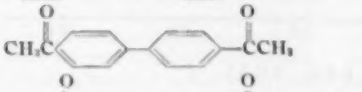
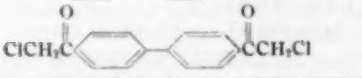
(*a*) For *in vitro* inhibition, acetyl-β-methylcholine (0.02 *M*) was employed as the substrate and Winthrop-Stearns bovine erythrocyte cholinesterase (11 units/cc.). Readings were taken for a two-hour period and all values were corrected for nonenzymatic hydrolysis. The 50% inhibitory levels of the compounds were calculated by the method of probits.

(*b*) The *in vivo* inhibition of dog's red blood cell and plasma cholinesterases was compared by using the substrates acetyl-β-methylcholine and benzoyl choline,[1] respectively. These experiments were conducted on 10-cc. samples of blood obtained through the use of a heparinized syringe followed by separation of the cellular elements through centrifugation at 3,400 r. p. m. for ten minutes. Both the cells and plasma were diluted 1:1 with Krebs-Ringer bicarbonate solution. Five-tenths cubic centimeters of diluted cells and 0.3 cc. of diluted plasma were used for all experiments. Total fluid volume of each Warburg flask was 3.0 cc. Blood samples were drawn at one-, five-, and fifteen-minute intervals following intravenous injection of the compound and the cholinesterase activity compared with cell and plasma controls.

(*c*) The *in vitro* inhibitions of dog's red blood cell and plasma cholinesterases were measured by adding

[1] The benzoyl choline was supplied through the courtesy of Dr. M. J. Schiffrin, Hoffmann-La Roche, Inc., Nutley, N. J.

TABLE II.—MISCELLANEOUS ANALOGS AND INTERMEDIATES

Compound No.	Structure	M. P., °C.	Analysis Calcd., %	Analysis Found, %
12		226–227	C 48.51 H 3.06	C 48.25 H 3.44
13		220	Br 22.60	Br 22.12
14		202–203	Ref. (199–200) (10)	
15		189–190	Ref. (189) (8)	
16		225–226	Ref. (226–227) (9)	

varying molar concentrations of the compound to the cells and plasma prepared as outlined under part *b*.

Isolated Gut Studies.—Strips of guinea-pig ileum of approximately 2.5 cm. in length were suspended in 30 cc. of oxygenated Tyrode's solution in an isolated tissue bath maintained at 38°. All drugs were dissolved in Tyrode's solution and injected in a constant volume.

Isolated Heart.—Oxygenated Locke-Ringer solution was perfused through hearts prepared according to the classical method of Langendorf. Drugs were dissolved in Locke-Ringer solution and injected into the coronary inflow cannula at such a rate that there was no alteration in inflow pressure.

Paralysis in Rabbits.—The procedure was essentially that of Chase, *et al.* (12), except for an increase of the interval between injections to thirty seconds.

RESULTS

Acute Toxicity.—A wide variation in toxicity was found for the compounds in this series. Table III lists the approximate LD_{50} found for various compounds following intraperitoneal injection. The approximate LD_{50} doses ranged from about 0.02 mg./Kg. for compound No. 11 to about 5.5 mg./Kg. for compound No. 3*a*. All mice died of respiratory paralysis as evidenced by the fact that upon opening the chest cavity the heart continued to beat. All fatal doses of the compounds produced death in about fifteen minutes except compound No. 11 which produced death in twenty to forty minutes. Compounds 1 through 5, as listed in Table III, produced marked salivation, urination, and defecation in the injected mice. This cholinergic response was studied more thoroughly using compound No. 3*a*.

TABLE III.—TOXICITY, CHOLINESTERASE INHIBITION AND GUT STIMULATION

Compound No.	Toxicity, Mg./Kg., in Mice[a]	Molar Concentration for 50% Cholinesterase Inhibition	Concentration in μg./30 Cc. Bath for 50% Contraction
1	1.5	8×10^{-10}	0.3
2	0.05	10^{-9}	0.4
3*a*	5.5	5×10^{-8}	...
3*b*	4.9	10^{-9}	0.4
4	4.5	2×10^{-7}	...
5	0.1	3×10^{-7}	16
6	2.0	5×10^{-6}	...
7	0.5	5×10^{-6}	200
8	1.0	4×10^{-6}	100
9	3.0	3×10^{-6}	200
10	0.8	6×10^{-5}	200
11	0.02	$<10^{-5}$	400

[a] The approximate LD_{50} was determined by using groups of ten mice.

In Fig. 1, dose-response curve *A* illustrates the toxicity for compound No. 3*a*. Dose-response curves *C* and *B* illustrate the potentiation in the LD_{50} for acetylcholine, as indicated through a lowering of the LD_{50} for acetylcholine from 260 mg./Kg. to 40 mg./Kg. following 0.4 mg./Kg. of compound No. 3*a*. The dose of 0.4 mg./Kg. of compound No. 3*a* in a control group of 30 mice did not produce any deaths.

Circulatory and Salivary Experiments.—The above potentiation of acetylcholine was studied in the dog. The dose-response curves illustrated in Fig. 2 show about a fifteenfold potentiation in the depressor response of acetylcholine following 1 μg./Kg. of compound No. 3*a*. After 2 μg./Kg. of No. 3*a*, there was about a thirty-eight fold potentiation in the depressor response of acetylcholine. The compound itself produced a typical muscarinic-type depressor response. On a molar basis, the compound was about three times as potent as acetylcholine in this latter regard. This response was blocked by atropine.

The dose-response curves concerning salivary flow are illustrated in Fig. 3 where a fifteenfold potentiation of acetylcholine at lower concentrations and about a fivefold potentiation at higher concentrations were realized.

Table IV illustrates the wide variation found in ability to potentiate the acetylcholine depressor response. Compound No. 11 exhibited no depressor response on the dog's blood pressure. With the mono quaternary derivatives (Nos. 13 and 14) only the *p*-phenyl phenacyl pyridinium compound (No. 13) indicated any degree of potentiation of acetylcholine on the depressor response. Compounds

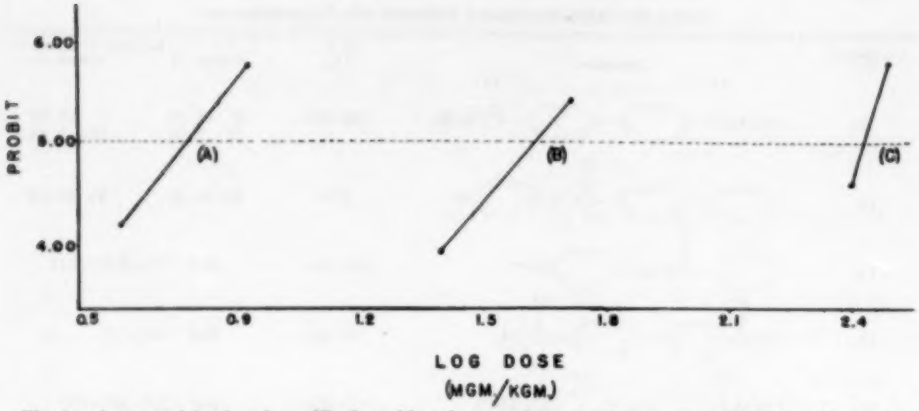


Fig. 1.—Acute toxicity in mice. (Each probit point was determined by a group of thirty mice.) *A.*—Dose-response curve for Compound No. 3*a*. *B.*—Dose-response curve for acetylcholine after 0.4 mg./Kg. of Compound No. 3*a*. *C.*—Dose-response curve for acetylcholine.

Nos. 1, 2, 3*a*, and 5 all seemed to produce tachyphylaxis upon repeated injection with respect to their own depressor effects. With compound No. 5 this phenomenon was especially noticeable.

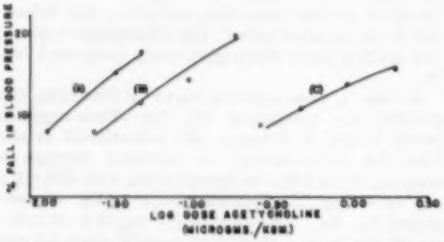


Fig. 2.—Depressor response to acetylcholine in the dog. *A.*—Dose-response curve for acetylcholine after 2 μg./Kg. of Compound No. 3*a*. *B.*—Dose-response curve for acetylcholine after 1 μg./Kg. of Compound No. 3*a*. *C.*—Dose-response curve for acetylcholine.

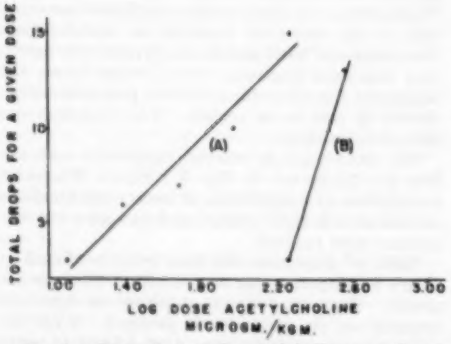


Fig. 3.—Potentiation of acetylcholine on salivary flow. *A.*—Dose-response curve for acetylcholine after 1 μg./Kg. of Compound No. 3*a*. *B.*—Dose-response curve for acetylcholine.

TABLE IV.—POTENTIATION OF THE DEPRESSOR RESPONSE OF ACETYLCHOLINE

Compound No.	Dose, Mg./Kg.	Potentiation
1	0.001	12-fold
2	0.001	12-fold
3*a*	0.001	15-fold
5	0.07	8-fold
13	0.10	5-fold
11	1.0	None
14	1.0	None[a]

[a] This compound produced a pressor response with a dose of 1 mg./Kg.

Cholinesterase Studies.—Several members of this series were found to be very potent inhibitors of "true" cholinesterase when studied manometrically. Table III lists the 50% inhibitory level of the various compounds using bovine erythrocyte cholinesterase.

Figure 4 illustrates a typical graph obtained by plotting μl. CO_2 *versus* time at varying molar concentrations of the inhibitor. Neostigmine bromide studied under similar conditions had a 50% inhibitory level of 8.5×10^{-9} *M*.

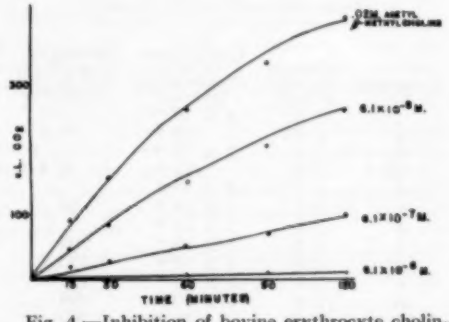


Fig. 4.—Inhibition of bovine erythrocyte cholinesterase by varying molar concentrations of Compound No. 3*a*.

A Lineweaver and Burk (13) plot of the data obtained using compound No. 3*a* is shown in Fig. 5. The lines representing varying molar concentrations of the inhibitor tend to converge on the ordinate indicating that this compound competitively inhibits bovine erythrocyte cholinesterase. Using compound No. 3*b* the *in vitro* 50% inhibitory level for dog cell cholinesterase was found to be approximately $10^{-9}M$. In contrast the 50% inhibitory level for plasma cholinesterase was found to be about 10^{-6} M. This indicates a wide variation in the ability of compound No. 3*b* to inhibit cell and plasma cholinesterase. Injections of 1 mg./Kg. of compound No. 3*b* intravenously in the dog did not show inhibition of either cell or plasma cholinesterase. With 3.0 mg./Kg. of compound No. 3*b* injected intravenously there was complete inhibition of the cell cholinesterase one minute following injection and no detectable inhibition of the plasma cholinesterase. In fifteen minutes the cell cholinesterase activity had returned to one-third of the control value.

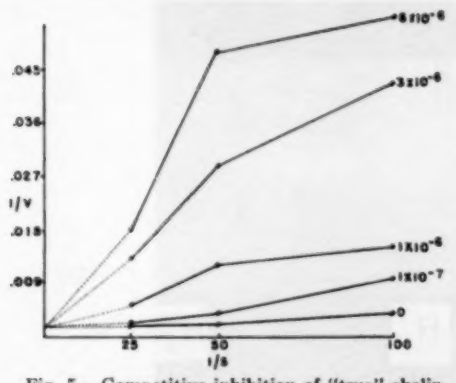


Fig. 5.—Competitive inhibition of "true" cholinesterase by Compound No. 3*a*. 1/V = reciprocal of μl. CO_2/two-hour period. 1/S = reciprocal of the molar concentration of acetyl-β-methylcholine. Concentration listed for each curve is the molar concentration of Compound No. 3*a*.

Isolated Gut Studies.—A summary of the data obtained on the guinea-pig ileum is shown in Table III. The ability of the various compounds to produce contractions in the isolated gut closely resembles their order of activity as cholinesterase inhibitors. The dose-response curves for the more potent cholinesterase inhibitors (Nos. 1 through 3*b*) were practically identical with that obtained for acetylcholine. When minimal stimulant doses of the compounds and acetylcholine were placed in the bath at the same time, only an additive response was obtained, indicating that none of the compounds potentiated acetylcholine in this preparation. This is in sharp contrast to the potentiating power of compounds Nos. 1 through 3*b* whose ability to potentiate acetylcholine's depressor response in the dog has been discussed. This failure to potentiate acetylcholine may be related to the high "pseudo" cholinesterase content of the gut (14). The more potent cholinesterase inhibitors produce a rather slow, irregular contraction. The compounds may be completely washed from the gut with a maximum of four washings as evidenced by the return of the gut to a state of relaxation and to control sensitivity to a test dose of acetylcholine.

Isolated Heart.—The marked ability of compound No. 3*a* to potentiate acetylcholine in this preparation is illustrated in Fig. 6. Following compound No. 3*a* the test dose of acetylcholine produced complete stoppage of the heart. Compound No. 3*a* (5γ) produced a prolonged slowing of the heart while 20 times this concentration would stop the heart. The slowing and stoppage of the heart was blocked by atropine. No effect on the coronary outflow was noticeable following injection of compound No. 3*a*. Compound No. 5 only indicated potentiation of acetylcholine at concentrations 10 times compound No. 3*a*. Compound No. 11 failed to show potentiation of acetylcholine even with concentrations as high as 1 mg.

Paralysis in Rabbits.—In rabbits the head drop dose (H. D. D.) for compound No. 3*a* was 5.4 mg./Kg., which is about 20 times the H. D. D. for *d*-tubocurarine chloride. Thirty μg./Kg. of neostigmine bromide three minutes preceding the injection of compound No. 3*a* significantly lowered the H. D. D. to 2.1 mg./Kg. ($P < 0.01$). Following the injection of compound No. 3*a* the rabbits salivated profusely for at least thirty minutes.

Special Observations on Compound No. 11.—Following 0.05 mg./Kg. intravenously in rabbits there were no observable symptoms for about ten minutes. After this period the rabbits appeared to be somewhat paralyzed in the hind legs. In about twenty minutes the rabbits began to have respiratory difficulty and alternated between periods of flaccidity and marked activity (spasms of the hind legs) without loss of consciousness. When death occurred it always followed in about forty-five minutes regardless of the dose. If the rabbit survived, as on a small dose (0.03 mg./Kg.), there were periods of depression and spasm for at least four to six hours. With controlled electrical stimulation of the sciatic nerve no diminution in foot twitch was noted even with a fatal dose of compound No. 11. In rabbits, this compound, therefore, did not appear to produce neuromuscular blockade. Its mechanism of action appeared to be central in locus and studies are now in progress to attempt to elucidate the exact site and type of involvement.

DISCUSSION

This class of compounds differs considerably from the series designed by Funke, *et al.* (7), in that the large, rigid, planar biphenyl nucleus tends to hold the quaternary nitrogens in an extended position of about 14 Å. as measured on Fisher-Hirschfelder-Taylor atomic models. With this rigid type of nucleus the statistical likelihood for an extended distance between the quaternary groups is much greater than in other series of bis quaternaries, e. g., those derived from chains of freely rotating bonds and in which only coulombic repulsive forces operate toward the extension of the molecules (15, 16).

One half of the parent structure (Compound No. 14) indicated only a nicotinic stimulant-type of response. The 4-phenyl derivative (Compound No. 13) possessed some anticholinesterase activity. This property may be due to a detergent action or an increase in molecular weight by lipophilic substitu-

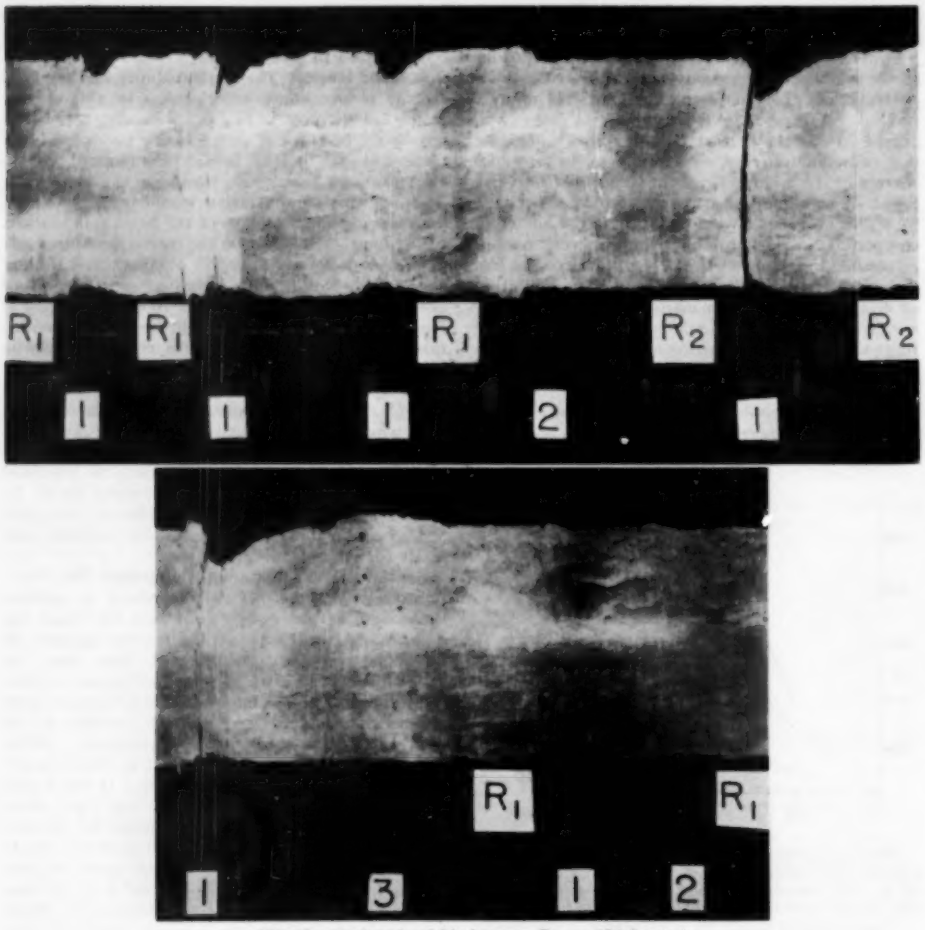


Fig. 6.—Isolated rabbit heart. R_1 = 120 beats/minute. R_2 = 110 beats/minute. (1) Represents the effect of 5 μg. of acetylcholine; (2) represents the effect of 5 μg. of Compound No. 3a; (3) represents the effect of 10 μg. of atropine sulfate.

tion. There is, however, another way of collating the activities of this series of materials with regard to their chemical structures.

Modern concepts concerning the structure of proteins emphasize regularity in the pattern of occurrence of amino acid residues with various proteins. More simply, the fact that the fundamental peptide chains are all derived from peptide bond formation through the α-carboxyl and α-amino groups insures a strict spacing of the emergent residues along the peptide chain.

The spacing between two peptide bonds itself is, therefore, a very regular distance commonly called the identity distance and is equal to 3.61 Å. (17). It is especially interesting that studies through the past decade relating various types of pharmacologic activity to intramolecular distances between groupings in various series of drugs have revealed repeatedly the identity distance or some whole number multiple thereof as the distance most readily correlated with optimum activity in given series. For example, the distance 14.5 Å. ($\backsimeq 4 \times 3.61$) used in the correlation of estrogenic activity (18) and curarimetic activity (19–21). The crucial distance for muscarinic and post ganglionic parasympathetic blocking agents is 7.23 Å. ($\backsimeq 2 \times 3.61$) (22).

Thus, if the multiple sites which bind a given drug are in reality the emergent residues of protein, one is not surprised that the distances collating drug activity follow the above indicated multiplicity rule with respect to the identity distance. It is the feeling of the authors, therefore, that the many examples of distance-activity correlation that have become apparent in recent years which are close multiples of the identity distance of protein is not fortuitous.

In the present case, distance between two quaternary nitrogens is a multiple of the identity distance

(4 × 3.61) and equal to the length of two acetylcholine molecules, e. g., as in succinyl dicholine (23). This may be an indication that the receptor site for the cationic end of acetylcholine is repeated in some regular fashion—perhaps in the form of a lattice work within the protein receptor surface.

In doubling the acetylcholine molecule a structure, succinyl dicholine, arises which (due to its double charge) may be bound more thoroughly to the receptor than acetylcholine, yielding a striking alteration in properties. Likewise the doubling of phenacyl pyridinium to give compound No. 3*a* yields a material with radically altered properties. The conjecture here is that the alteration in properties achieved by the doubling of phenacyl pyridinium (Compound No. 14) is due to an increased stability arising out of dual attachment. There is yet at least one other hypothesis which may account for the alteration in activity, however, that of an increase in "umbrella effect" or "adumbrating" action[1] achieved through doubling the molecule. In the present series, at least, such an increase in steric hindrance through increase in molecular weight alone does not appear to suffice in the interpretation of the actions of the present series of materials. Thus, the 4-phenyl derivative (Compound No. 13) which represents a sizable increase in the steric factor is yet very low in activity in comparison with the *bis* quaternary analog (Compound No. 3*a*). It is possible, however, to interpret the action of the *bis* quaternary as an increase in steric inhibition of the acetylcholine receptor site, provided two-point binding is assumed. Thus, if two-point binding of the quaternary nitrogens is assumed over a distance on the acetylcholine receptor equivalent to two times the length of acetylcholine then the intermediate esteratic site would be screened from the entrance of

[1] Both of these terms are used by the authors Pfeiffer (22), Loewe and Harvey (24) to describe the concept of steric hindrance.

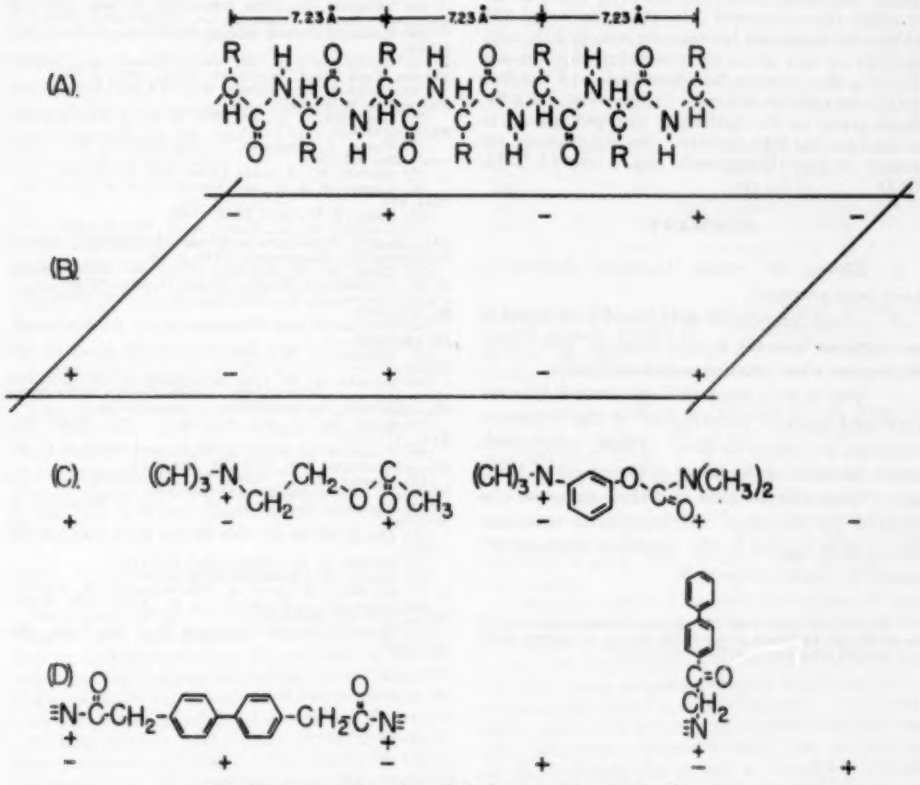


Fig. 7.—Illustration of possible drug-receptor interactions.

(*A*) Section of protein illustrating those emergent charged groups which are located at double the "identity" distance from one another.

(*B*) Oblique view of the protein surface indicating spacings of charged *R*-groups which constitute the hypothetical receptor.

(*C*) Interaction of acetylcholine and neostigmine with cationic (−) and esteratic (+) receptor sites.[a]

(*D*) Illustration of dual attachment by compound No. 3*a* with resultant screening of the esteratic (+) site for acetylcholine to be compared with one point attachment for the relatively inactive compound No. 13.

[a] The terms cationic and esteratic sites are terms employed with the same connotation as used by Wilson, *et al.* (25).

acetylcholine and an inhibition of acetylcholine hydrolysis would result. These relationships are shown in Fig. 7.

In this class of compounds the substitution on the quaternary nitrogen plays a major role in their activity. While most other potent anticholinesterase compounds have methyl or ethyl substitution on the quaternary nitrogen, optimum activity in this series was shown by a heterocyclic nitrogen substitution.

Initially in this class of compounds as in the other series of cholinesterase inhibitors previously mentioned there appeared to be a relationship between the toxicity and anticholinesterase activity. The activities of the dimethylethanol derivative (Compound No. 11) indicates that this relationship in the present series is not realized. The peripheral nervous system is not markedly depressed by this material and this compound appears to kill by action on the central nervous system. In laboratory animals, the symptoms produced by Botulinus toxin resemble closely the toxic symptoms of this compound both with regard to time of onset and general gross appearance. Botulinus antitoxin, however, failed to antagonize this compound (No. 11) in mice, but this failure of antagonism between the drug and the antitoxin in no way alters the possibility of a common site of action between Botulinus toxin and the drug within the nervous system. In this series, the β-hydroxy group on the quaternary nitrogen appears to be essential for high toxicity. See, for example, the desoxy analogs (Compounds Nos. 8 and 10, Table III).[3]

SUMMARY

1. Eleven *bis* onium biphenyl derivatives have been prepared.

2. Three compounds were found to be equal to neostigmine bromide as inhibitors of "true" cholinesterase when studied manometrically.

3. The more potent cholinesterase inhibitors produced marked potentiation of the depressor response to acetylcholine. These compounds alone produced a depressor response equal to or larger than acetylcholine and their response was blocked by atropine. Tachyphylaxis was produced with regard to the depressor response effected by these compounds.

4. Gut stimulation by the compounds themselves paralleled their *in vitro* cholinesterase inhibitory potency, but none of the compounds produced potentiation of acetylcholine-induced contractions of the guinea-pig ileum.

5. The potentiation of acetylcholine in the isolated heart seems to parallel the *in vitro* cholinesterase inhibition.

6. One of the materials showed high toxicity (LD_{50} = 0.02 mg./Kg.) in white mice on intraperitoneal injections. In rabbits, 0.05 mg./Kg. intravenously produces a slow insidious death, probably of central origin.

7. Mechanism of action and structure-activity relationships were discussed.

[3] Preliminary tests with derivatives of compound No. 11 in which the hydroxyl group is blocked as an acetate ester or a methyl ether bear out this conjecture.

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The Effect of Ascorbic Acid and Trace Elements on Vitamin B_{12} Assays*

By E. M. STAPERT, E. B. FERRER, and L. STUBBERFIELD†

A study was made to determine the effects of certain trace elements and ascorbic acid upon vitamin B_{12} activity in aqueous solutions. Four of these trace elements combined with ascorbic acid decreased the vitamin B_{12} activity as demonstrated by microbiological assay. Copper combined with ascorbic acid was the most active. The chemical data show destruction of vitamin B_{12} in the presence of copper and ascorbic acid. Potassium cyanide and sodium diethyldithiocarbamate, materials known to complex copper, are effective in preventing destruction of vitamin B_{12}.

SEVERAL PAPERS have been published concerning the effect of ascorbic acid upon cyanocobalamin and hydroxocobalamin (1–6). We encountered inconsistent results in the U. S. P. Microbiological Assay for vitamin B_{12} (7) when the product under test contained ascorbic acid. There was some indication these inconsistencies could be due to trace impurities in the water used as a solvent in the assay procedure. This prompted us to investigate the effect of certain trace elements on the assay of vitamin B_{12} in the presence of ascorbic acid.

METHODS

Microbiological.—A procedure similar to our regular assay technique was employed in making the following studies. Combinations of materials to be studied were blended for five minutes in a Waring Blendor with 300 cc. of water and then assayed by the U. S. P. Microbiological Assay for vitamin B_{12} (7), modified by determining growth turbidimetrically after twenty hours' incubation.

Chemical.—Aqueous solutions of vitamin B_{12} were saturated with ammonium sulfate and extracted with *n*-butanol (8). The *n*-butanol solution was separated and extracted with distilled water. The absorbancy of the aqueous extract was measured at 361 mμ and the concentration of vitamin B_{12} determined from the specific absorbancy (9).

EXPERIMENTAL

Destruction of Oral Grade Vitamin B_{12} Activity by Ascorbic Acid and Copper.—Solutions of 12.5 and 25.0 μg. oral grade vitamin B_{12} produced by fermentation and high in hydroxocobalamine were prepared. These solutions were blended for five minutes in the presence of combinations of ascorbic acid, copper, potassium cyanide, and sodium diethyldithiocarbamate as shown in Table I. The results by microbiological assay are expressed as per cent recovery of vitamin B_{12}.

It is evident from the results in Table I that ascorbic acid, copper, potassium cyanide, and sodium diethyldithiocarbamate alone do not reduce vitamin B_{12} activity, but ascorbic acid and copper in combination greatly reduce it. Potassium cyanide and sodium diethyldithiocarbamate will prevent the destructive effect of ascorbic acid and copper upon vitamin B_{12} activity.

TABLE I.—THE EFFECT OF ASCORBIC ACID AND COPPER ON THE ACTIVITY OF ORAL GRADE VITAMIN B_{12}

Oral Grade Vit. B_{12}, μg.	Ascorbic Acid, Gm.	Copper, P. P. M.	KCN or $(C_2H_5)_2NCSSNa$, Mg.	Vitamin B_{12} Recovery, %
12.5	...	..	...	106.8
25.0	...	..	...	103.6
12.5	0.825	..	...	98.0
25.0	1.65	..	...	100.0
25.0	...	10	...	91.6
25.0	...	..	100 KCN	98.4
25.0	1.65	10	...	60.0
				58.0
12.5	0.825	..	100 KCN	106.8
25.0	1.65	10	100 KCN	102.0
25.0	...	10	100 KCN	97.0
25.0	...	..	257 $(C_2H_5)_2NCSSNa$	94.0
25.0	1.65	10	257 $(C_2H_5)_2NCSSNa$	90.4

Destruction of U. S. P. Vitamin B_{12} by Ascorbic Acid and Copper.—The above experiments were repeated with 20 μg. U. S. P. vitamin B_{12} in place of oral grade vitamin B_{12}. The microbiological assay results are shown in Table II and are comparable to those obtained in the previous experiments, except that the loss of vitamin B_{12} activity was not as great when treated with ascorbic acid and copper.

Cooperman (10) has reported that potassium cyanide has a stabilizing or enhancing effect upon the activity of vitamin B_{12} when assayed microbiologically. We were therefore interested in determining the effect when potassium cyanide was added to the combinations listed in Tables I and II. The results show that, in the presence of ascorbic acid and copper, potassium cyanide protects the activity of U. S. P. vitamin B_{12} as well as oral grade vitamin B_{12}. Preliminary studies indicate that the copper may combine with the potassium cyanide and form a complex which does not destroy the vitamin B_{12} activity. Sodium diethyldithiocarbamate acts in a similar manner.

The Effect of Various Concentrations of Copper.—Since the above experiments demonstrated that 10 p. p. m. of copper in the presence of ascorbic acid decreased the vitamin B_{12} activity, it seemed desir-

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TABLE II.—THE EFFECT OF ASCORBIC ACID AND COPPER ON THE ACTIVITY OF VITAMIN B_{12} (U. S. P.)

Vit. B_{12} U. S. P., μg.	Ascorbic Acid, Gm.	Copper, P. P. M.	KCN, Mg.	Vit. B_{12} Recovery, %
20	...	..	...	109.5
20	1.65	..	...	97.5
20	...	10	...	106.0
20	...	..	100	107.5
20	1.65	10	...	74.3
20	1.65	..	100	104.5
20	1.65	10	100	106.0
20	...	10	100	106.5

able to determine the effect of varying amounts of copper when constant amounts of U. S. P. vitamin B_{12} (20 μg.) and ascorbic acid (1.65 Gm.) were used. Copper was used in concentrations from 0.1 to 1,000 p. p. m. The sample preparation was the same as described above and the microbiological assay results are given in Table III.

The results in Table III show a progressive decrease in vitamin B_{12} activity as the amount of copper is increased. This is significant in view of the fact that many multivitamin products which are assayed for vitamin B_{12} contain quantities of copper and ascorbic acid comparable to some of the amounts listed. A control which contained 1,000 p. p. m. copper without ascorbic acid resulted in full recovery of the U. S. P. vitamin B_{12}.

TABLE III.—THE DESTRUCTION OF VITAMIN B_{12} ACTIVITY BY ASCORBIC ACID WITH VARIOUS CONCENTRATIONS OF COPPER

Copper, P. P. M.	Vitamin B_{12} Recovery, %
0.0	97.5
0.1	87.7
1	90.4
2	82.5
3	82.5
4	77.0
5	77.0
6	74.0
7	84.5
8	73.0
9	72.0
10	67.6
100	53.8
1000	35.6

Extraction and Spectrophotometric Determination of U. S. P. Vitamin B_{12} Solutions.—A solution of U. S. P. vitamin B_{12} was prepared, containing 8.79 μg./cc. as determined spectrophotometrically (9). Ten cubic centimeters of this solution, made up to 60 cc., was transferred to a low actinic 125-cc. separator with the addition of 10 cc. of *n*-butanol. Sufficient ammonium sulfate (solid) was added and the separator was shaken vigorously at intervals until the aqueous phase was saturated with ammonium sulfate. The *n*-butanol phase was separated, cen-

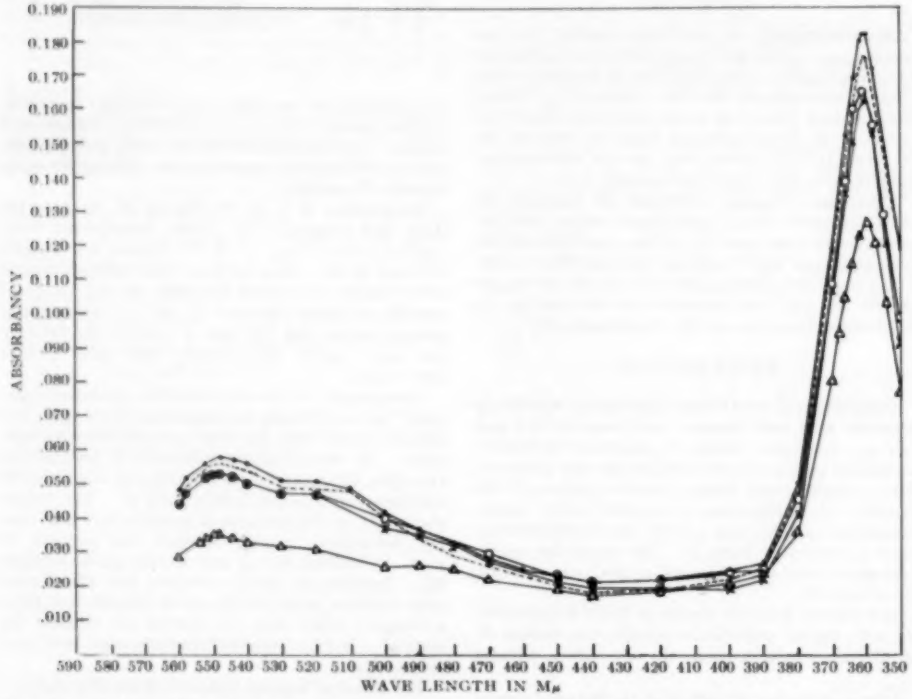

Fig. 1.—*a* (●——●) Standard aqueous B_{12} solution; *b* (- - -) B_{12} solution extracted; *c* ○——○ B_{12} plus ascorbic acid blended and extracted; *d* (△——△) B_{12} plus ascorbic acid and copper blended and extracted; *e* (X——X) B_{12} plus ascorbic acid, copper and KCN blended and extracted.

trifuged, and a 5-cc. aliquot was extracted with 5 cc. of distilled water (2 minutes' vigorous shaking). This was centrifuged and the aqueous phase transferred to a 1-cm. Beckman cuvette. A blank was prepared by treating 60 cc. of distilled water in the same manner. The absorbancy was determined at intervals from 500 mμ to 350 mμ with the use of a Beckman model DU spectrophotometer and compared with the absorption curve of the original vitamin B_{12} solution. This is shown by curves *a* and *b* in Fig. 1. The extraction effected a sixfold concentration of vitamin B_{12} with 96.2% recovery. The extraction technique separated the vitamin B_{12} from reaction products and enabled the experiments to be carried out at concentrations of vitamin B_{12} comparable to those found in usual assay solutions. The solubility of *n*-butanol in water did not affect the absorption curves as long as the blanks were handled identically with the experimental solutions.

Verification of Recovery by Extraction Method.—Five cubic centimeters of vitamin B_{12} solution, containing 55.3 μg./cc. (microbiological assay, 55.4 μg., 56.4 μg.) was made up to 60 cc. and extracted as previously described. The recovery with a sixfold increase in concentration was 93.5%. The *n*-butanol extract was assayed microbiologically and found to contain 26.1 μg./cc. of vitamin B_{12} activity. These results are comparable in that approximately 95% of the vitamin B_{12} is recovered when a sixfold concentration is affected by the extraction. Microbiological assay value of the final aqueous phase was 26.7 μg./cc. or 95.4% recovery.

Chemical Determination of the Destructive Action of Ascorbic Acid and Copper on Vitamin B_{12}.—One hundred cubic centimeters of solution containing 175.88 μg. vitamin B_{12} and 0.55 Gm. ascorbic acid was blended in a Waring Blendor for five minutes. Copper, 10 p. p. m., was added to 100 cc. of an identical solution and treated in the same manner. A third solution identical with the second was made up with the addition of 33 mg. potassium cyanide. This was also blended for five minutes. Appropriate blanks were prepared and a 50 cc. aliquot of each solution was extracted with the use of 10 cc. *n*-butanol followed by 1:1 extraction of the *n*-butanol with water. The spectra are shown by curves *c*, *d*, and *e* in Fig. 1. The vitamin B_{12} concentration used was such that the final concentration would be 8.79 μg./cc. provided there was complete extraction. The absorbancies represented by curves *c*, *d*, and *e*, therefore, can be compared with the absorbancy of curve *b*. This shows that under the conditions of treatment, ascorbic acid alone caused destruction of 5.7% of the vitamin B_{12}, while ascorbic acid and copper caused destruction of 27.4% of the vitamin B_{12}. The addition of potassium cyanide prevented this destruction (7.4%). Examination of the curves show that ascorbic acid and copper affect the visible portion of the spectrum, absorption ratio 361 mμ/548 mμ = 3.63, while the addition of potassium cyanide not only prevents destruction of vitamin B_{12}, but restores this portion of the spectrum, absorption ratio 361 mμ/548 mμ = 3.06 (9).

Effect of Blending Time on Destruction of Vitamin B_{12} by Ascorbic Acid and Copper.—The same experiment was carried out except that the blending time was ten minutes. In this experiment 34.3% of the vitamin B_{12} was destroyed by ascorbic acid and copper.

The Effects of Other Trace Elements and Ascorbic Acid upon Vitamin B_{12} Activity.—Many pharmaceutical products which contain vitamin B_{12} and ascorbic acid also contain various trace elements; it was therefore considered important to determine the effect of some of these elements on vitamin B_{12} activity when assayed microbiologically. Vitamin B_{12} U. S. P. (20 μg.) and ascorbic acid (1.65 Gm.) were added to 300 cc. of water which contained 10 p. p. m. of each of the elements listed in Table IV. These mixtures were blended for five minutes and then assayed microbiologically. The results are shown in Table IV.

TABLE IV.—THE EFFECT OF ASCORBIC ACID AND CERTAIN TRACE ELEMENTS ON VITAMIN B_{12} ACTIVITY

Element	Vitamin B_{12} Recovery, %
Cobalt ($CoSO_4$)	100.0
Magnesium ($MgSO_4$)	101.0
Iodine (KI)	103.0
Iron ($FeSO_4$)	99.2
Zinc ($ZnSO_4$)	94.5
Manganese ($MnSO_4$)	87.5
Fluorine ($CaFl_2$)	86.7
Molybdenum (Na_2MoO_4)	86.0

Manganous, fluoride, and molybdate ions show some destructive effect on vitamin B_{12} under the conditions of this test, while cobaltous, magnesium, iodide, ferrous, and zinc ions show no effect. Since these elements may be found in multivitamin products they could influence the microbiological assay for vitamin B_{12}.

SUMMARY

The data presented here show that the stability of vitamin B_{12} in solutions containing ascorbic acid is influenced by certain trace elements. Copper was the most effective element tested, and as little as 0.1 p. p. m. caused appreciable destruction of vitamin B_{12}, when solutions were agitated in a Waring Blendor. The combination of copper and ascorbic acid had greater destructive action on vitamin B_{12b} than on vitamin B_{12}, but in both cases this action was prevented by the addition of potassium cyanide or sodium diethyldithiocarbamate. Molybdate, fluoride, and manganous ions also caused destruction of vitamin B_{12} in solutions containing ascorbic acid while cobalt, magnesium, iodide, ferrous, and zinc ions did not. None of the ions when tested separately affected the stability of vitamin B_{12} in solution.

Spectrophotometric data on identically treated solutions verified the amount of destruction caused by ascorbic acid and copper and also the prevention of this destruction by potassium cyanide. It is suggested that these factors be taken into consideration when assaying vitamin B_{12} by the U. S. P. method.

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The Effect of Freeze-Drying on the Glycosidal Content of *Digitalis purpurea* and *Digitalis lutea* L.*

By F. P. COSGROVE† and E. P. GUTH‡

A study of the effects of freeze-drying on the glycosidal content of the leaves of *Digitalis purpurea* L. and *Digitalis lutea* L. has been made. The results indicate that freeze-drying is applicable as a means of drying leaves of these two species of digitalis. It is suggested that this method of drying be adopted for leaves of *Digitalis purpurea* that are to be used as a source of material for the official preparations of this drug. The moisture content of the lyophilized material compares favorably with that found for the corresponding oven-dried samples. In regards to the toxicity tests, it was found that no significant differences were present with samples made from either the lyophilized or oven-dried material. The chemical assays indicate notable differences in the majority of samples compared. The types and quantities of glycosides present in the powdered samples are not under investigation.

HAMILTON (1) has shown that fresh leaves of *Digitalis purpurea* have greater activity than dried leaves, but it is not always convenient to use the fresh leaves immediately after harvesting.

Wijngaarden (2) has reported that in moist digitalis leaves, enzymes cause a slow but steady loss of activity. Stoll and Kreis (3) pointed out clearly the need of inactivating the digitalis enzymes. Studies by King and Gisvold (4) and Youngken, *et al.* (5), have shown that freezing temperatures preserve the potency of the constituents of *Digitalis purpurea* leaves.

In light of the literature which points to the destructive action of the enzymes in digitalis leaves as well as the disagreement concerning the optimum time and temperature required to dry these leaves, it follows that a method which could remove moisture at low temperatures would be an ideal method for drying this drug.

One process of desiccation used to dry material at freezing temperatures is called freeze-drying. Chambers and Nelson (6) have shown that freeze-drying is applicable for drying belladonna leaves. With this process the moisture in the material is frozen and removed by means of reduced pressure directly from the frozen state to the vapor state.

Although few data are available on the chemical composition of the constituents of *Digitalis lutea*, it has been used in place of the official digitalis and appears to be almost identical in activity with it. Leone (7), Boriani (8), and Welti (9) studied the activity of *Digitalis lutea* and found it to be equal to *Digitalis purpurea*. White and Morris (10), as well as Konnerth and Pickering (11), found that *Digitalis lutea* grown in Minnesota was equal in activity to *Digitalis purpurea*.

The objectives of this investigation are to determine the effects of freeze-drying on the glycosidal content of *Digitalis purpurea* L. and *Digitalis lutea* L., and to compare these results with those

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obtained with the leaves of the same source which were oven-dried at 50° for thirty-six hours.

EXPERIMENTAL

The materials used in this investigation were the leaves from *Digitalis purpurea* L., 1st and 2nd year plants, and *Digitalis lutea* L., 2nd and 3rd year plants.

The leaves were collected at random from the Drug Garden of the College of Pharmacy, The Ohio State University. Leaves from each of the plants were divided into two lots, *A* to be oven-dried at 50° for thirty-six hours, and *B* to be lyophilized at 250 μ of pressure and a temperature of less than 35°. All samples to be lyophilized were packed in dry ice immediately after harvesting in order to prevent, as far as possible, any change in the constituents by the enzymes or other reactants.

Samples of *Digitalis purpurea* L., 1st and 2nd year plants, and *Digitalis lutea* L., 2nd and 3rd year plants, were oven-dried at 50° for thirty-six hours. Samples of *Digitalis purpurea* L., 1st and 2nd year plants, and *Digitalis lutea* L., 2nd and 3rd year plants, were lyophilized by means of the Stokes Freeze Drying Equipment Model 2004-L.

The dried material was ground to a No. 40 powder and placed until used in tightly closed, light-resistant bottles, which contained a small bag of Drierite.

Moisture Content.—In order to determine whether the samples met the official requirements for moisture content, the powders were assayed according to the U. S. P. XIV method for material containing no principles volatile at 100°. The freeze-drying process gave results comparable with those obtained with the oven-drying procedure. Results are given in Table I.

TABLE I.—PERCENTAGE OF MOISTURE[a]

Sample	Age of Plant, Years	Method of Drying	Percentage of Moisture
D. purpurea	1	Oven[b]	4.4
D. purpurea	1	Freeze-dried[c]	4.2
D. purpurea	2	Oven[b]	4.6
D. purpurea	2	Freeze-dried[c]	5.4
D. lutea	2	Oven[b]	3.7
D. lutea	2	Freeze-dried[c]	5.4
D. lutea	3	Oven[b]	5.0
D. lutea	3	Freeze-dried[c]	5.0

[a] As determined by the Brabender Semi-Automatic Moisture Tester, Rochelle Park, N. J.
[b] Oven-dried at 50° for thirty-six hours.
[c] Lyophilized at 250 μ of pressure at a temperature of less than 35° by means of the Stokes Freeze Drying Equipment Model 2004-L.

Extraction and Determination of Total Glycosides.—To extract the total glycosides without subjecting them to possible destruction by heat, the cold percolation method was employed. A 10-Gm. sample of each drug was packed in an 80-ml. percolator and macerated for twenty-four hours with 95% alcohol. Continuous percolation was carried out at a rate of 1 ml. per minute until a portion of the percolate gave a negative Molisch test and a high transmittancy reading on an electrophotometer. Alcohol, U. S. P. was added to the percolate collected from each sample to bring the total volume to 1500 ml.

The total glycoside content present in the percolate was determined by the modified Knudson-Dresbach method (12). A 25-ml. aliquot was concentrated spontaneously to approximately 5 ml. in order to eliminate possible interference from the ethanol. The determinations were made with the aid of a Fisher electrophotometer at 525 mμ using a 23-ml. absorption tube, and readings were compared with those obtained with the U. S. P. Digitalis Reference Standard tincture. The total glycosides expressed as U. S. P. units of Reference Standard are given in Table II.

TABLE II.—TOTAL GLYCOSIDES EXTRACTED FROM 10 GRAMS OF POWDERED DRUG

Sample	Age of Plant, Years	Method of Drying	No. of Determinations	Average Total Glycosides as Units of Reference Standard[c]
D. purpurea	1	Oven[a]	6	15.5
D. purpurea	1	Freeze-dried[b]	6	13.8
D. purpurea	2	Oven[a]	6	6.1
D. purpurea	2	Freeze-dried[b]	6	7.4
D. lutea	2	Oven[a]	5	14.7
D. lutea	2	Freeze-dried[b]	5	12.9
D. lutea	3	Oven[a]	6	15.8
D. lutea	3	Freeze-dried[b]	6	13.2

[a] Oven-dried at 50° for thirty-six hours.
[b] Lyophilized at 250 μ of pressure at a temperature of less than 35° by means of the Stokes Freeze Drying Equipment Model 2004-L.
[c] As determined by the Knudson-Dresbach method (12).

Glycosidal Content of Sample Tinctures.—Tinctures were prepared from the lyophilized and oven-dried material according to the official directions and assayed for total glycosides by the modified Knudson-Dresbach method (12). Readings were made as described above. The results expressed as percentage potency of Reference Standard are shown in Table III.

TABLE III.—GLYCOSIDAL CONTENT OF SAMPLE TINCTURES

Sample	Age of Plant, Years	Method of Drying	No. of Determinations	Average Potency of Reference Standard[c]
D. purpurea	1	Oven[a]	9	119.8
D. purpurea	1	Freeze-dried[b]	9	111.2
D. purpurea	2	Oven[a]	9	74.7
D. purpurea	2	Freeze-dried[b]	9	69.7
D. lutea	3	Oven[a]	6	102.0
D. lutea	3	Freeze-dried[b]	6	94.5

[a] Oven-dried at 50° for thirty-six hours.
[b] Lyophilized at 250 μ of pressure at a temperature of less than 35° by means of the Stokes Freeze Drying Equipment Model 2004-L.
[c] As determined by the modified Knudson-Dresbach method (12).

TABLE IV.—EVALUATION OF TOXICITY TESTS OF SAMPLE TINCTURES ON FROGS

Sample	Age of Plant, Years	Method of Drying	No. of Frogs Used	Range of Systolic Stoppage Times[c]	Average Systolic Stoppage Times[c]	Standard Error	Degrees of Freedom	t Value	At 95% Level
D. purpurea	1	Oven[a]	15	22–48	31.1	1.92	28	0.546	2.048
D. purpurea	1	Freeze-dried[b]	15	26–47	32.5	1.70			
D. purpurea	2	Oven[a]	18	25–66	46.4	2.92	34	1.113	2.032
D. purpurea	2	Freeze-dried[b]	18	24–82	50.6	4.21			
D. lutea	3	Oven[a]	17	24–59	43.8	2.82	32	0.244	2.048
D. lutea	3	Freeze-dried[b]	17	25–79	42.7	3.50			

[a] Oven-dried at 50° for thirty-six hours.
[b] Lyophilized at 250 μ of pressure at a temperature of less than 35° by means of the Stokes Freeze Drying Equipment Model 2004-L.
[c] Times are in minutes.

Toxicity Tests.—The toxicity of the samples was determined by diluting the tinctures to 1% with physiological salt solution and using the cup infusion method (13). While this method measures chiefly the toxicity reaction of digitalis, it gives satisfactory results for comparing whole leaf preparations such as tinctures and infusions.

The time required to produce systolic stoppage of the frog's heart was used as the basis for the toxicity of the samples. Results are given in Table IV.

Evaluation of the Toxicity Results.—The results of the toxicity tests were evaluated statistically. The t values were calculated as proposed by Snedecor (14), by pooling the variance of the results of the tinctures made with oven-dried material with the variance of the results of the corresponding tinctures made from lyophilized products using appropriate degrees of freedom, and testing the hypothesis that the means of the two samples being compared are the same. The t values that are greater than 95% probability t value, at the appropriate degrees of freedom, are considered significant. Results are given in Table IV.

DISCUSSION

The results of all the colorimetric assays with the exception of those for total glycosides of *Digitalis purpurea*, 2nd year plants, indicate that the oven-dried samples contain supposedly higher content of glycosides than the corresponding lyophilized material. While this possibility might exist, the results of the toxicity tests when evaluated statistically indicate that no significant differences were present among the samples compared.

No explanation is given for the behavior of *Digitalis purpurea*, 2nd year plants, other than to state that many of the leaves contained small brown spots and the plants were flowering at the time of harvesting.

A variation of the amount of primary and secondary glycosides may be a possible reason for differences in the majority of samples compared colorimetrically. Bell and Krantz (15) and Eastland, *et al.* (16), have shown that secondary glycosides and aglycones give greater optical density to the color reaction of this test than do primary glycosides when equal concentrations of these constituents are compared. Therefore, it seems that the degree of complexity of the glycoside mixture, namely the relative proportions of primary glycosides to secondary glycosides and aglycones, has an important effect on the colorimetric assay. If primary glycosides are broken down to secondary glycosides and aglycones by heat and enzymatic action, then this may partly explain why the oven-dried samples show supposedly higher glycosidal content when assayed by the modified Knudson-Dresbach method.

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Phenol Coefficients and the Ferguson Principle*

By NASEEM A. ALLAWALA† and SIDNEY RIEGELMAN‡

It is shown that the thermodynamic activity at which many phenols produce an equal degree of antibacterial action is approximately the same. The thermodynamic activity of a dissolved phenol can be expressed in terms of the per cent saturation by setting the saturated solution as the standard state of reference. Thus it is demonstrated that the equitoxic solutions of different phenols are those in which the thermodynamic activity or the per cent saturation and not the actual concentrations are the same. It is shown that, as expected, the reported phenol coefficients of 23 different phenols examined are approximately the same as the ratios between the solubility of the unknown disinfectant and the solubility of phenol and that the deviations are interpretable.

AFTER EXAMINING the narcotic and disinfectant action of alcohols, Ferguson arrived at the principle that the toxicities should be compared with the values of the thermodynamic activities of the toxic substances and not with the values of the concentrations of the toxic agents (1). This principle has been further substantiated by the study of relative effectiveness of a large series of narcotics by Brink and Posternack (2) and by the study of factors governing the permeability of the insect cuticle by Webb (3). One of the basic requirements for the validity of the Ferguson principle seems to be that the toxic action under study be independent of time or that it be an equilibrium type and not a kinetic type action (1, 2). Even though antibacterial action is evaluated under conditions of fixed time (such as is done in the phenol coefficient method), the results are not time-independent because the kinetic phenomena of diffusion, adsorption, and penetration are involved and may not have reached a condition of steady state. However, an analysis of the term availability revealed that even though the killing of bacteria does not constitute an equilibrium-type action, the Ferguson principle may be expected to hold for certain types of antiseptic action (4).

Many biologically active compounds seem to act by accumulating at some vitally important site of the bacterial cell. They obviously owe their activity to their property of favorable penetration of the cell wall or of favorable adsorption at this cell site. Correlations have been made between this biological activity and the oil/water partition coefficient, the surface tension reducing property, or some other physical property of different compounds in a homologous series. Since these physical properties have never been measured in the active cellular biophase, but instead in artificial media, they are open to criticism.

Assuming an equilibrium or a steady state condition, it should be possible to overcome this impasse. By expressing the concentrations in terms of the thermodynamic activities, we obtain a value proportional to the biologically active concentration in that part of the cell in which the molecular events are actually occurring. In such a system of molecules, the partial molal free energy ($\bar{F}_x$) of the constituent, x, has the same value in *all* phases of the system. Since an absolute scale of partial molal free energies has not been established, it is customary to select one specific state as the standard state of reference, thus resulting in a standard free energy, $\bar{F}_x^0$, and in effect measuring the difference in partial molal free energies. The thermodynamic activity, a_x, is related to this difference in the following manner: $\bar{F}_x - \bar{F}_x^0 = RT \ln a_x$. At constant temperature and pressure the value of $\bar{F}_x^0$ will be constant and the natural logarithm of the activity becomes a measurement of the partial molal free energy.

The value of the activity will, of course, depend upon the choice of the standard state for each substance tested. It is a customary procedure in thermodynamic investigations to select a standard state so that at infinite dilution $a_x/m_x = 1$. While such a procedure is logically simpler for electrolytes, there is no theoretical or physical requirement for this selection, especially in considering nonelectrolytes. When a solution is sufficiently dilute so that Henry's Law is a useful approximation, one need not define the standard state in terms of an infinitely dilute solution. One is equally justified in setting the pure substance as the standard state. By this alternative selection, we are able to compare the compounds on a basis of the easily measured, physically significant, water-solubility values.

The question, therefore, involves deciding on whether the solution is sufficiently dilute to allow

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convenient use of this approximation. It has been experimentally found that except for solutions of electrolytes and for solutions of high polymers in solvents of low molecular weight, we normally expect the dilute solution laws to be good to at least 1 per cent at concentration running from zero to 0.1 mole/liter and good to a few per cent up to 1 mole/liter (5). Fortunately, biological concentrations are sufficiently dilute to fall in the former category.

Ferguson (1) and Brink and Posternack (2) found that the thermodynamic activity at which many substances produce equal degrees of biological activity is approximately constant. Upon selecting the pure substances as the standard state, it is proposed that the antibacterial activity of a series of phenols should be approximately equal to a constant per cent saturation of the active constituent. The purpose of this paper is to point out the extent of applicability of this concept to a portion of the existing information and to new bacteriological data.

EXPERIMENTAL PROCEDURES

Preparation of Saturated Solutions.—Saturated solutions of phenols were prepared by allowing excess of solute to stand in 0.5 *M* Sorenson buffer (*p*H 7.4) in a 25° thermostat for over a month (with occasional shaking). All dilutions were made with the same buffer.

Determination of 99% Killing Time.—Freshly prepared spores of *Bacillus cereus* (*metiens*) were used, and the method described by Marks, Wyss, and Strandskov (6) for I_3-KI systems were followed with the following exceptions: the final test solution was adjusted to contain 10^8 spores per cc. (99% killing time corresponded to 10^4 surviving spores per cc.) and no sodium sulfite was used.

Cell Suspension Method.—The F. D. A. phenol coefficient procedure given by McCulloch (7) was followed with certain important modifications. Instead of a broth culture, a standardized cell suspension was used as the inoculum. The cell suspension was made as follows: 0.5 cc. of a twenty-four-hour culture of *Micrococcus pyogenes* var. *aureus* was poured into 15 cc. of hardened agar in 125-cc. Erlenmeyer flasks. After twenty-four hours (± 10 minutes required for manipulation of the several flasks), the surface of the agar was washed with 10 cc. of 0.5 *M* Sorensen buffer *p*H 7.4. The suspension was centrifuged at 4,000 r. p. m. for fifteen minutes. The supernatant fluid was discarded and the centrifuged cells resuspended in 100 cc. of buffer, resulting in a cell concentration of approximately 30 million cells per cc. It should be emphasized that the 0.5 cc. of broth inoculum contains as many as 300 million cells. In the test procedure adopted, the use of a more concentrated cell suspension resulted in a significant variation in the number of bacteria per inoculum. Since this affected the toxic concentration, a highly diluted cell suspension was selected so that one million cells per cc. could be obtained in the final test solution (1 cc. of the cell suspension diluted to 30 cc. with the test solution).

EXPERIMENTAL RESULTS AND DISCUSSION

Even though the extension of the Ferguson principle to the solutions of solubilized antimicrobial agents was found to be quite useful in correlating the antibacterial activity with the amount of bactericidal agent (4, 8), it seems necessary to provide other experimental evidence for the extension of the Ferguson principle to other biological systems. There are several ways in which this extension has been verified, four of which are discussed below.

Structural Modification.—If the Ferguson principle is applicable under the kinetic conditions, then the addition of a methyl group in an homologous series may make the compound more effective on the weight basis, but should not change its antibacterial activity on the basis of thermodynamic activity. Ferguson (1, 9) and Brink and Posternack (2) have reported data on several homologous series which follow the Ferguson principle. Even though most of the series of compounds studied by them were not antibacterial agents, the discussion presented by them offers theoretical justification for the principle.

Growth Curve.—From the Ferguson principle it follows that the ratio between the antibacterial effectiveness of various agents (on weight basis) should be the same as the ratio of their saturation solubilities in the test medium. Vincent and other workers (10–14) have studied the bacteriostatic influence of the esters of 4-hydroxy benzoic acid on the growth of several microorganisms. From analysis of growth curves, the inhibitory effectiveness of these esters was determined. In all cases, the degree of inhibition caused by an ester was found to be inversely related to its solubility. Since the experiments lasted over a long period (several hours) such a correlation is indeed remarkable.

99% Killing Time.—Since all solutions in equilibrium with excess solid have the same thermodynamic activity, then by the Ferguson principle, all saturated solutions should show the same bactericidal activity. In this laboratory the 99% killing time for *B. cereus* (*metiens*) spores was determined using saturated solutions of twenty-three different phenols. All of the phenols gave approximately the same killing time; however, the 99% killing time observed was too long (more than twenty-nine hours) to allow any accurate determinations.

Phenol Coefficient.—The phenol coefficients of compounds related to phenol in their mechanism of action should be the same as the ratio of their saturation solubilities—if the Ferguson principle is valid. Ferguson (1) suggested a graphical method for ascertaining whether or not a series of compounds follows the principle. This method involves plotting the log of the toxic concentration (for any given action) against the log of the solubility. If a straight line can be drawn through the data for the series of compounds under study, then a correlation between the thermodynamic activity and the biological action is indicated. This test can be applied to the phenol coefficient data (*B. typhosus*) of Schaeffer and Tilley (15) on an homologous series of *p*-*n*-alkyl phenols whose solubilities were subsequently deter-

mined by Lamson, *et al.* (16). Figure 1 represents both the biological toxic concentration and the saturation concentration in mole/liter. It will be noted that a linear correlation is found. The dashed line, representing the saturated solution values on both the ordinate and abscissa, is useful in explaining the apparent lack of activity (under the test procedure) of the *p*-hexyl phenol on several different strains of *B. typhosus* (Table 6, ref. 15) and the highly variable data of *p*-amyl phenol. Schaeffer particularly notes that the saturated solution of hexyl phenol failed to kill the test organism in fifteen minutes on four of the five strains tested. This cutoff of activity indicates, as originally pointed out by Ferguson, that if the saturated solution falls below a given activity (i. e., is below a certain equitoxic concentration) the compound should be inactive. Utilizing the data of various workers (17–19), Fig. 2 (upper graph) represents the correlation of the molar solubility of a series of phenols with their reported phenol coefficients (*M. pyogenes* var. *aureus*), the latter values being expressed in terms of their molar toxic concentrations. Although the deviations are quite large, a correlation appears to exist. Unfortunately, insufficient attention has been paid to the method of preparation of stock solution of the active test drugs. The investigators have frequently followed one of the following procedures for the poorly soluble compounds: (*a*) in preparing the stock solutions, the compounds have been dissolved in alcohol or other water-miscible organic solvents and diluted therefrom. (*b*) Alkaline compounds have been used to dissolve the test drug. The use of sodium carbonate, for example, would convert a phenol into the water-soluble phenolate and leave only a fraction of the drug in the active, undissociated form. (*c*) A suspension or emulsion of the test drug has been used as the stock "solution." (*d*) A supersaturated solution has been prepared by heating the suspension or emulsion of the compound. Certain phenols have been noted to form supersaturated solutions which are exceedingly difficult to reduce to normal solubility values (20). It should be noted that methods (*b*) and (*c*) above were suggested by Ewe (21) to overcome the variation in phenol coefficients obtained on poorly soluble compounds.

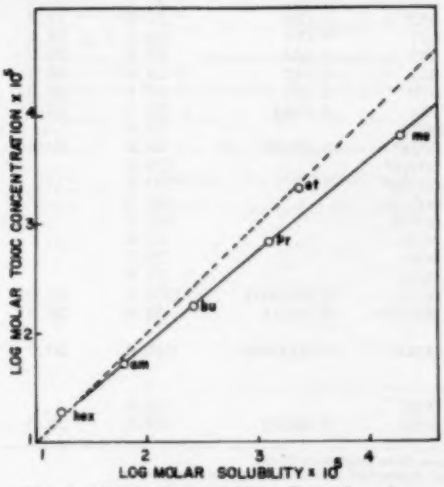

Fig. 1.—Toxic (bactericidal—*B. typhosus*) concentration *vs.* water solubility for a homologous series of *p*-*n*-alkyl phenols. Dashed line indicates water saturation concentrations.

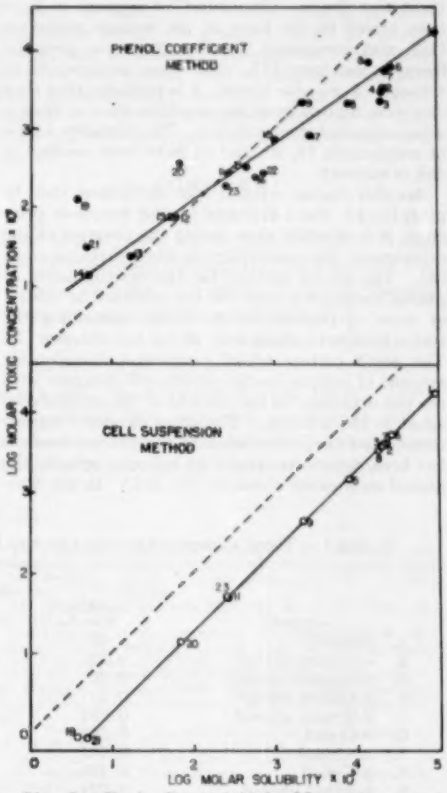

Fig. 2.—Toxic (bactericidal—*M. pyogenes* var. *aureus*) concentration *vs.* water solubility for a series of phenols. Dashed line indicates water saturation concentrations. (See Table I for number identification of each compound.)

The solubility values of the compounds represented in Fig. 2 (upper graph) are from several sources and they are not known with equal certainty. However, it is most probable that the deviations from the linear representations are due to one of the above variations in preparing the test solutions. To a certain extent, examination of the source references confirms this postulate (17–19). The saturation solubility concentrations (dashed line) indicate that compounds 12 through 15 and 19 through 21 appear to show activity in contradiction to the Ferguson postulate. Table I lists the composite data for the molar solubility, toxic concentration, and per cent saturation of a series

of phenols as determined by the phenol coefficient and cell suspension method. Column five represents the toxic concentration, obtained from the phenol coefficient determinations, in terms of the per cent saturation of the solution. With the exception of compound 20, all of the above-mentioned compounds appear to have been tested as supersaturated solutions. Unfortunately, scant information is available in the original articles as to the method of preparation of the solution for each of these test drugs. Compound 19 appears to have been tested in the form of the sodium phenolate (19), and compound 20 as a dispersion prepared from an emulsion (17, 22). Since compounds 12 through 15 are also liquids, it is probable that they also were diluted from the emulsion state or from a supersaturated stock solution. The solubility values of compounds 19, 20, and 21 have been verified in this laboratory.

Another factor causing the deviations can be postulated. For adherence to the Ferguson principle, it is essential that during the progress of the experiment, the availability should remain constant (4). The official method for the determination of phenol coefficients calls for the addition of 0.5 cc. or more of peptone broth culture containing the test organism to about 5 cc. of the test solution (7). The broth culture added contains a considerable amount of organic matter which will compete with the test organism for the amount of the antibacterial agent in the solution. The effect of added organic matter and the constitution of the culture medium has been shown repeatedly to influence greatly the phenol coefficients obtained (19, 23).[1] In the solutions with low capacity and low solubility, the net effect of the addition of organic matter will be the lowering of the thermodynamic activity leading to a lower phenol coefficient. Therefore, during the present research the antibacterial activities were determined by use of a standardized cell suspension in water instead of the broth culture. The lower curve of Fig. 2 represents the correlation between the molar solubility (thermodynamic activity) and the molar toxic concentration as determined on the standardized bacterial cell suspension. It is apparent that the correlation has been greatly improved and by the modifications of the test procedure even the most insoluble compound tested possesses sufficient concentration to give biological activity.

As shown in column 6 of Table I, the saturated solutions of all phenols had to be diluted approximately to the same extent in order to kill a standardized suspension of *M. pyogenes* var. *aureus* in ten minutes but not in five minutes. Since all saturated solutions have exactly the same thermodynamic activity, the final solutions after dilution also had the same thermodynamic activity, and, therefore, a direct correlation between the thermodynamic activity and the biological action has thus been provided. If the relative antibacterial

[1] The published information on this subject seems to be confined to the effect of added organic matter to the peptone broth, and Dr. C. M. Brewer of the F. D. A. in a personal communication was kind enough to inform us that, "I cannot recall, nor has a fairly diligent search of the literature indicated, that resuspended organisms have been used in phenol coefficient testing. At least, any publication of such work is unknown to me."

TABLE I.—TOXIC CONCENTRATIONS AND THE PER CENT SATURATION OF A SERIES OF PHENOLS

	Compound	Solubility, Mole/L.	Toxic Concentration, Mole/L.		Per Cent Saturation[d]	
			Phenol Coeff.[a]	New[c]	Phenol Coeff.	New
1.	Phenol	0.96	0.175	0.175	18.2	18.2
2.	*o*-Chloro phenol	0.22	0.034	0.040	15.4	18.2
3.	*m*-Chloro phenol	0.20	0.022	0.037	11.0	18.2
4.	*p*-Chloro phenol	0.21	0.032[b]	0.038	15.2	18.2
5.	*p*-Bromo phenol	0.081	0.021	0.015	25.9	18.2
6.	*o*-Cresol	0.269	0.062	0.054	23.0	20.0
7.	*m*-Cresol	0.231	0.055	0.042	23.8	18.2
8.	*p*-Cresol	0.185	0.070	0.034	37.8	18.2
9.	*p*-Ethyl phenol	0.024	0.022	0.0043	92.0	18.2
10.	*p*-*n*-Propyl phenol	0.010	0.0075	...	75.0	...
11.	*p*-*n*-Butyl phenol	0.0027	0.0024[b]	0.00053	89.0	20.0
12.	*p*-*n*-Amyl phenol	0.0006	0.00076[b]	...	126.0	...
13.	*p*-*n*-Hexyl phenol	0.0001	0.00025[b]	...	250.0	...
14.	*p*-*n*-Heptyl phenol	0.00005	0.00014	...	280.0	...
15.	*p*-Benzyl phenol	0.00054	0.00076	...	140.0	...
16.	Thymol	0.0060	0.0025	...	41.6	...
17.	2,4-Dichloro phenol	0.028	0.008	...	28.5	...
18.	2,4,6-Trichloro phenol	0.0046	0.0034	...	74.0	...
19.	Pentachloro phenol	0.000037	0.0012	0.0000094	3250.0	25.0[e]
20.	2-Hydroxy-5-chloro diphenylmethane	0.00069	0.00036[b]	0.00014	52.0	20.0
21.	2,2'-Dihydroxy-3,5,6,-3',5',6'-hexachloro diphenylmethane	0.00005	0.00032	0.0000098	640.0	20.0
22.	2,4-Dibromo phenol	0.0075	0.0030	...	40.0	...
23.	Hexyl resorcinol	0.0026	0.0180	0.00052	69.0	20.0

Values for the phenol coefficients and water solubilities are taken from references 17–19.
[a] Reported phenol coefficient (*Micrococcus pyogenes* var. *aureus*) converted into molar toxic concentration.
[b] Average of several literature values.
[c] Cell suspension method (see Experimental Procedures).
[d] The toxic concentration expressed in terms of per cent of the water-saturated solution.
[e] Since Pentachloro phenol is quite acid ($pK_a = 5.3$), only a fraction of the dissolved molecules are in the active, undissociated form.

effectiveness (RAE) is defined as the ratio between the thermodynamic activity of the solution of an unknown disinfectant and the thermodynamic activity of the solution of phenol which will kill the test organism in a given time, then all of the phenols tested above have about the same RAE.

The data listed in Table I confirm the statement that the equitoxic solutions of different toxic substances are those in which the thermodynamic activities and not the absolute concentrations are the same. In the above solutions of phenols even though the concentrations may vary over a thousandfold, the thermodynamic activity as determined by the dilution of the saturated solution remains the same.

It has long been recognized that the action of hypnotics, general anesthetics, and some of the milder insect poisons is not related to the presence or absence of some particular chemical group, but depends rather on certain physical properties which make for accumulation. These substances have been called (24) structurally nonspecific agents in contrast to the structurally specific agents such as sulfonamides or 8-hydroxy quinolines.

From the above experimental data on phenolic compounds one might, at first thought, come to the conclusion that these phenols are active against bacteria in a structurally nonspecific manner. At present there is no evidence that the majority of phenols show competitive or noncompetitive inhibiting action on an essential enzymatic process, and according to Gale and Taylor (25) phenol acts like a surface-active agent in causing cytolysis, measurable by the release into the surrounding medium of individual amino acids (glutamine, arginine, lysine). Nevertheless, further experiments should be conducted before conclusive statements are made concerning the specificity or nonspecificity of structure in phenols.

It might be well to point out that an interesting additional extension of the Ferguson principle to the structurally specific agent is a possibility. While maintaining the structurally specific groups, it should be valuable to modify chemically other structurally nonspecific groups in order to investigate whether variation in biological activity thereby produced may be related to equivalent variation in their thermodynamic activity. This, of course, suggests the use of saturated solution or their equivalent dilutions for the biological test procedure.

SUMMARY

1. The correlation between the thermodynamic activity and the antibacterial action has been substantiated through the application of the Ferguson principle to a series of phenols varying in their phenol coefficients from 1 to 675.

2. By use of a cell suspension method and carefully prepared saturated solutions, it has been shown that the solutions of these phenols must be 18–20 per cent saturated in order to kill a standardized suspension of *M. pyogenes* var. *aureus* in ten minutes but not in five minutes.

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Synthesis and Evaluation of a New Ganglionic Blocking Agent*

By F. W. SCHUELER and H. H. KEASLING

Details concerning the synthesis and pharmacologic evaluation of a new hexamethonium-type ganglionic blocking agent containing a "built-in" muscarinic moiety are described. The inclusion of this latter moiety may be regarded as an attempt to increase parasympathetic tone toward the alleviation of such undesirable side actions as constipation and mydriasis produced by hexamethonium.

IN A PREVIOUS report by Schueler and Keasling (1), it was noted that the substance β-trimethylammonium - (β′ - trimethylammonium ethyl) propionate dibromide(I) and its homolog β - triethylammonium - (β′ - triethylammonium ethyl) propionate dibromide(II) held a pharmacologic relationship with respect to ganglionic activity analogous to the simple homologous ions tetramethylammonium(III) and tetraethylammonium(IV). The chemical structural and pharmacologic relationships are shown in Table I.

The agents I and II (Table I) are interesting from another standpoint. They are related to hexamethonium(V) in the sense of being bisquaternaries of similar (N to N) length.

TABLE I.—STRUCTURAL RELATIONSHIPS BETWEEN SOME DRUGS ACTING ON GANGLIA

Ganglionic Stimulant	Ganglionic Depressant
I. $(CH_3)_3\overset{+}{N}CH_2CH_2OC(=O)CH_2CH_2\overset{+}{N}(CH_3)_3$	II. $(C_2H_5)_3\overset{+}{N}CH_2CH_2OC(=O)CH_2CH_2\overset{+}{N}(C_2H_5)_3$
III. $\overset{+}{N}(CH_3)_4$	IV. $\overset{+}{N}(C_2H_5)_4$
	V. $(CH_3)_3\overset{+}{N}CH_2CH_2CH_2CH_2CH_2CH_2\overset{+}{N}(CH_3)_3$

In the light of preliminary tests (1) on these materials it was conjectured that compound II (Table I) effected a more profound depression of sympathetic ganglia than of parasympathetic ganglia. There is, however, another hypothesis which is worthy of consideration. This hypothesis is the following: Compound II blocks sympathetic and parasympathetic ganglia equally well but, in addition, produces a low degree of postganglionic parasympathetic stimulation, thus effecting an over-all relatively greater depression of the sympathetic nervous system than of the parasympathetic nervous system.

The lines of argument supporting this hypothesis are twofold. First, there is no satisfactory evidence, either morphologic or functional for believing that a difference exists between the accessibility or general responsiveness of sympathetic and parasympathetic ganglia. Second, from a chemical structural standpoint, compounds I and II (Table I) possess the features of a "doubled" muscarinic moiety (2). A pharmacologic correlate with this latter idea is the fact that both I and II effect depressor responses which are potentiated by neostigmine (1).

Whatever may be the interpretation of the actions of compound II in regard to its effectiveness in depressing sympathetic tone beyond that of parasympathetic tone, certain clinically useful potentialities are clear. Namely, compound II is a ganglionic blocking agent which may avoid the side effect liabilities of hexamethonium that are referable to concomitant depression of parasympathetic tone, e. g., constipation and mydriasis.[1] The objective, therefore, of the present article is to report the details of synthesis and pharmacological evaluation of compound II as a ganglionic blocking agent.

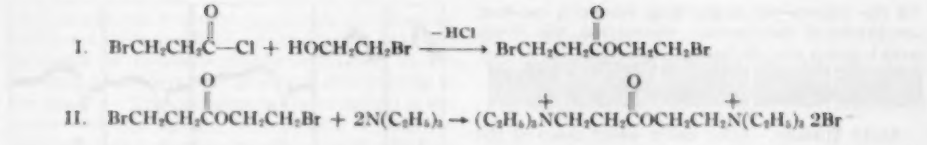
MATERIALS AND METHODS

Synthesis.—The preparation of β-triethylammonium-(β′-triethylammonium ethyl) propionate dibromide (compound II of Table I and hereafter designated RACET) follows from a two-step procedure according to the scheme on the next page.

The Preparation of $BrCH_2CH_2OC(=O)CH_2CH_2Br$.—One mole of pure ethylene bromohydrin is allowed to flow into 1 mole β-bromopropionyl chloride. After adding about 1/2 mole of the ethylene bromohydrin, the flask (under reflux) is heated gently to induce the reaction. (The flask and reflux should be arranged so that the flask may be warmed or cooled

* Received July 23, 1953, from the Department of Pharmacology, College of Medicine, State University of Iowa, Iowa City, Iowa.

[1] Preliminary clinical trial in about 20 cases of essential hypertension have thus far borne out the validity of this conjecture.



rapidly for proper control of the reaction.) After the evolution of HCl is well under way, more of the ethylene bromohydrin may be added from time to time to sustain a steady reaction. After final addition of ethylene bromohydrin, the mixture is allowed to stand overnight. The next day dry air is blown through it to eliminate the excess of HCl gas which remains. After the air treatment the crude ester should be free from the odor of HCl. The elimination of excess HCl is necessary to drive the reaction to completion, thereby securing the highest possible yield of the ester. Heat should not be used as it tends to discolor the ester (probably through dehydrohalogenation) and leads to the formation of contaminating unsaturated esters.

The ester is then distilled under vacuum in an all-glass apparatus with a long distillation column. A Claisen flask may be used but a long Vigreux column is better. The fraction boils at 108–111°/5 mm. It is collected and distilled again. The yield at this point is 60%. It may be increased to 70 or 75% if the other fractions are carefully and repeatedly distilled.

Anal.—$C_5H_8O_2Br_2$.—Calcd. for Br 61.48; Found: Br 62.06. Density: 1.8168 30/30.

The pure ester is a colorless, highly refracting liquid which keeps well in a dark bottle in the cold, but becomes gradually yellow at room temperature in the light.

This ester has some slight irritant properties so that due care must be exercised in its handling.

The Preparation of β-Triethylammonium-(β′-triethylammonium ethyl) Propionate Dibromide (Hereafter Referred to as RACET).—Twenty-nine gram, (0.1 mole) portion of the dibromide ester (from I above) is added to a tenfold (volume) excess of dry pure triethylamine. The mixture is allowed to stand overnight during which time about 50% of the theoretical quantity (based on the ester) of RACET crystallizes. This product is filtered off and washed three times with dry ether by mixing with 100 ml. portions and filtration. The product is then air dried till free from the odor of ether or triethylamine. Rewashing with dry ether is necessary if any odor remains. Pure ether should be used because peroxides may cause some coloring by oxidation of bromide ion to bromine. The recovered triethylamine may be recharged with another portion of ester, and this process can be repeated. The yield is 95% if the mixture of amine and ester is allowed to stand two weeks or more or is refluxed gently on the water bath after removal of the initial crop of crystals.

RACET[2] is a white crystalline solid (nonhygroscopic). It is soluble in water and in alcohol, insoluble in ether, benzene, or chloroform. It sublimes slowly at 163° as fine feathery needles, m.p. 244° (corr.). Calcd. for $C_{17}H_{38}Br_2N_2O_2$. N, 6.06; Found: 6.31.

Pharmacologic Methods—Acute Toxicity.—Toxicity experiments were carried out on white mice following intraperitoneal administration of the material in physiological saline.

Evaluation as a Ganglionic Blocking agent.[3]—*Effect upon Postural Hypotension.*—Normal dogs anesthetized with 36 mg./Kg. of pentobarbital sodium quickly recovered from the slight drop in blood pressure (carotid arterial recording) induced by rapid tilting (legs lowered) to a head elevation of 45°. Surgical sympathectomy, adrenolytic drugs, and some ganglionic blocking agents so alter the reflex control that the tilting procedure results in a definite postural hypotension.

Effect as an Antagonist to Tetramethylammonium (TMA).—Dogs anesthetized with pentobarbital sodium 36 mg./Kg. were prepared for recording of the carotid arterial blood pressure and treated with alternate doses of TMA and RACET.

Effect upon the Carotid Sinus Reflex Response.—In these experiments dogs anesthetized with 36 mg./Kg. of pentobarbital sodium were prepared through cannulation of the femoral artery for blood pressure recording. The vagi were sectioned and 0.5 mg./Kg. of atropine injected intravenously. Both carotid arteries were isolated and the effect upon ganglionic function evaluated as follows. First, the normal response to successively increasing time intervals of double carotid occlusion was determined. This procedure was then repeated after successive intravenous (femoral vein) doses of RACET.

Effect upon the Cervical Sympathetic Ganglion.—Cats were anesthetized with 0.75 cc./Kg. of Dial-Urethane (Ciba) administered intraperitoneally. The cervical sympathetic nerve was stimulated with supermaximal shocks by means of a Grass Model S4A stimulator. The drugs were administered in 0.9% saline via the femoral vein. The activity of the nictitating membrane was recorded by means of a pulley lever system writing upon a smoked drum.

Effects upon the Isolated Heart.—Isolated rabbit hearts were prepared for perfusion with oxygenated Ringer-Locke solution according to the classical Langendorf procedure. Drugs dissolved in the Ringer-Locke solution were injected via the coronary inflow cannula under such a pressure that the inflow pressure was maintained constant. Records of the amplitude and coronary flow, by a tipping bucket system, were obtained kymographically.

Effects upon the Isolated Gut.—Rabbit ileum strips, approximately 3 cm. in length, were suspended in a muscle cup of 50-cc. capacity arranged for ready renewal of the bath medium which consisted of oxygenated Tyrode's solution. After a suitable period

[2] The authors are indebted to the Barlow-Maney Laboratories for the preparation of the sterile solution and capsules of RACET which were used throughout these and the aforementioned clinical studies.

[3] Tetraethylammonium was used in a number of similarly designed experiments together with its hydrazonium analog (3).

for the adjustment of gut strip toward a constant amplitude of spontaneous contraction, the drugs were injected into the bath.

RESULTS AND DISCUSSION

Acute Toxicity.—Mice under lethal doses of the drug RACET showed no evidence of salivation or lacrimation but after five to ten minutes exhibited fine tremors, followed by clonic convulsions, leading to death by respiratory failure. The heart showed a slow, weak beat upon immediate opening of the chest cavity following respiratory failure. The LD_{50} is greater than 500 mg./Kg. as indicated in Table II.

TABLE II.—MOUSE ACUTE TOXICITY OF RACET

Dose, Mg./Kg.	No. Mice Used	Lethal
300	20	1
400	20	4
500	20	8

When the abdominal cavities of mice killed by RACET were examined, no apparent gross depression of peristalsis was noted when comparison was made with normal decapitated controls.

Tests Concerning Ganglionic Blockade.—*Effects upon Postural Hypotension.*—Figure 1 illustrates the typical effects of RACET upon the readjustment of a dog to postural hypotension. One notes the increasing difficulty in readjustment to posturally effected alterations in blood pressure.

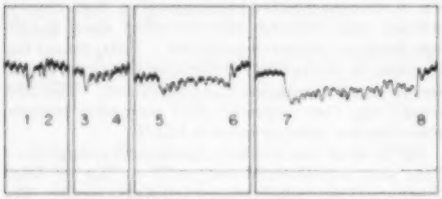


Fig. 1.—Positional hypotension.—(1) Tilt 45° head elevation; (2) return to horizontal; (3) tilt to 45° head elevation after 4 mg./Kg. RACET; (4) return to horizontal; (5) tilt to 45° head elevation after 8 mg./Kg. RACET; (6) return to horizontal; (7) tilt to 45° head elevation after 16 mg./Kg. RACET; (8) return to horizontal.

Effect as an Antagonist of TMA.—That RACET is quite capable of antagonizing the effects of a well-known ganglionic stimulant tetramethylammonium (TMA) is illustrated in Fig. 2. Successive doses of RACET progressively inhibit the pressor response to TMA until only the depressor effect of this latter agent is readily apparent. However, the continued repeated administration of TMA without the intermediate injection of RACET eventually yields a marked pressor response, comparable with that observed upon the animal untreated with RACET. The base blood pressure as such, however, does not return to normal. Thus, RACET may be antagonized to some degree by sufficiently high doses of TMA in so far as the acute pressor response of this latter agent is concerned. These results may be interpreted as a competition between TMA and RACET for ganglionic receptors.

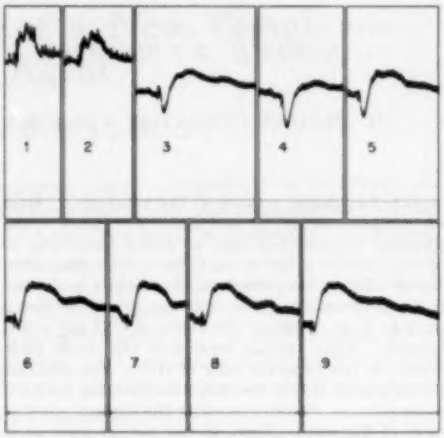


Fig. 2.—Competitive action with TMA.—(1) 0.5 mg./Kg. TMA; (2) 0.5 mg./Kg. TMA after 0.5 mg./Kg. RACET; (3) 0.5 mg./Kg. TMA after 8 mg./Kg. RACET; (4) 0.5 mg./Kg. TMA after 16 mg./Kg. RACET; (5) 0.5 mg./Kg. TMA; (6) 0.5 mg./Kg. TMA; (7) 0.5 mg./Kg. TMA; (8) 0.5 mg./Kg. TMA; (9) 0.5 mg./Kg. TMA.

Doses 5 through 9 of TMA were administered to exhibit the reversibility of the blockade established by RACET.

Effect upon the Carotid Sinus Reflex Response.—The effect of RACET upon successively longer periods of carotid occlusion are illustrated in Fig. 3. It was of interest to investigate these results by the graphical methods of Lineweaver and Burk (4).

Thus, if the concentration of acetylcholine produced in ganglia is proportional to the time of occlusion and the subsequent pressor effect produced is in turn related to the effectiveness of the acetyl-

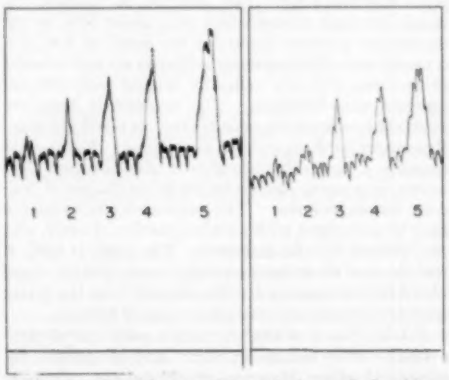


Fig. 3.—Double carotid occlusion.—Left side—Control. Right side—Following 2 mg./Kg. of RACET. 1–5 second double occlusion. 2–10 second double occlusion. 3–15 second double occlusion. 4–20 second double occlusion. 5–30 second double occlusion.

choline released, it should be possible to determine whether a given ganglionic blocking agent acts competitively or noncompetitively in relation to the blockade of endogenously produced acetylcholine in the ganglia. Thus, by plotting the reciprocal of the pressor response against the reciprocal of occlusion time following increasing dosage levels of the blocking agent, a double reciprocal plot of lines characteristic of competitive inhibition is obtained. Table III exhibits the primary data obtained in such an experiment while Fig. 4 illustrates the type of competitive effects observed.

TABLE III.—THE EFFECT OF GRADED DOSES OF RACET UPON THE CAROTID REFLEX PRESSOR RESPONSE

Carotid Occlusion Time (Sec.)	Drug Mg./Kg. 0 Mm.	2 Mm.	4 Mm.	8 Mm.	16 Mm.
5	6	4	2	1	0
10	14	10	9	5	1
15	22	20	18	11	3
20	27	24	24	18	8
30	30	28	25	22	15
45	..	..	..	..	18

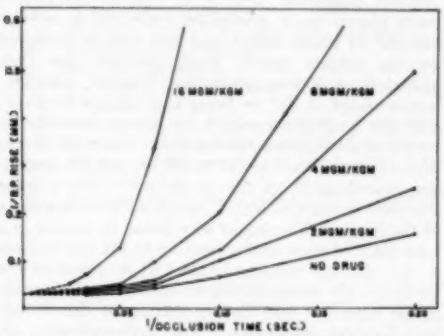


Fig. 4.—Double reciprocal plot of Table III. Drug doses for curves refer to RACET.

Effect upon the Cervical Sympathetic Ganglion.—Using three cats it was found that doses of from 2 to 4 mg./Kg. suffice to block the response of the contraction of the nictitating membrane of the cat under supermaximal preganglionic stimulation of the cervical sympathetic nerve.

Effects upon the Isolated Heart.—That RACET in relatively high doses is capable of evoking an increase in the amplitude of the isolated heart is illustrated in Fig. 5. RACET does not, however, effect an increase or decrease in either heart rate or coronary flow. It does, in relatively high doses, depress the effect of acetylcholine in this preparation. The hypotensive effect of RACET, therefore, does not appear to involve any degree of depression in heart conduction or contractility.

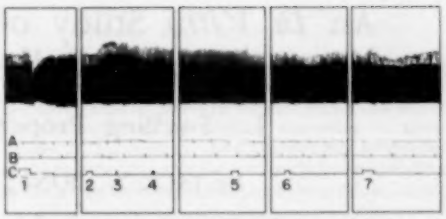


Fig. 5.—Isolated heart perfusion. (*A*) 4 ml. tipping bucket for coronary flow. (*B*) Time 60 seconds. (*C*) Base line and drug injections. (1)—5 μg. acetylcholine. (2)—1 mg. RACET. (3)—5 μg. acetylcholine. (4)—5 μg. acetylcholine. (5)—5 μg. acetylcholine. (6)—5 μg. acetylcholine. (7)—10 μg. acetylcholine.

Effects upon the Isolated Gut.—In concentrations of 1:10,000 RACET may effect some increase in the amplitude or responsiveness to acetylcholine of the isolated intestine.

CONCLUSION AND SUMMARY

1. RACET is a ganglionic blocking agent with a potency roughly in the same range as tetraethylammonium.

2. Like tetraethylammonium, RACET antagonizes competitively the ganglionic stimulation effected either through the administration of tetramethylammonium or by acetylcholine liberated endogenously through reflex stimulation.

3. In so far as studies upon the isolated heart are concerned, RACET probably does not effect its hypotensive action through any depression of heart rate or amplitude, nor does it effect any significant alteration in coronary flow.

4. RACET effects no depression in the amplitude of contraction of the isolated intestine or responsiveness to acetylcholine stimulation. It may, on the other hand, possess a slight degree of stimulating action.

5. In the light of the above findings, chemical structural features, and results developed in a previous report (1), it is conjectured that RACET may be considered as a hexamethonium-type ganglionic blocking agent, possessing a "built-in" muscarinic moiety. It appears, however, that the degree of its action in this latter respect is merely sufficient to overcome the loss in parasympathetic tone which it produces as a ganglionic blocking agent.

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An *In Vitro* Study of Various Commercially Available Bulk-Type Laxatives*,†

I. Swelling Properties and Gel Textures

By JACK N. BONE‡ and L. WAIT RISING§

A study of the swelling properties and gel textures in distilled water, sodium chloride solution, hydrochloric acid solution, sodium bicarbonate solution, artificial gastric juice, and artificial intestinal juice has been made on 24 commercially available bulk-type laxatives representing the various types now on the market. The data collected are presented.

ONE of the more important and numerous classes of pharmaceuticals is the bulk laxative. The bulk-type laxative acts by increasing the volume of the contents of the gastrointestinal tract, and this increase in volume acts as a stimulus for defecation. Most of the bulk-type laxatives are hydrophilic and because of their water-binding properties produce a soft consistency of the stool.

Gray and Tainter (1) classified the colloid laxatives into six groups, namely, colloidal clay, dried fruits, marine, acacia, tragacanth, and psyllium. Since this classification was made, many new bulk laxatives have appeared on the market. Holbrook (2) has observed the behavior of carob gum in the gastrointestinal tract of man and has found that, although this hemicellulose is similar to that of psyllium and tragacanth, it has certain characteristics which would cause it to fall into a separate group. There has been considerable publicity and use of bulk laxatives composed of methylcellulose. Therefore, these two groups, carob and methylcellulose, may be added to the above classification.

Some of the properties of bulk laxatives which determine their efficacy are swelling, not only the extent but also the time of swelling, and the texture of the mass formed. Considerable work has been done in evaluating bulk laxatives by observing their effects upon the stool and by observing their passage through the gastrointestinal tract by means of X-ray studies (3–13). The number of published investigations concerning *in vitro* evaluation of bulk laxatives is not so great (1, 14). This study was undertaken in order to make available additional comparative data upon the swelling properties and gel textures of the various commercially available bulk-type laxatives.

PROCEDURE

Swelling Properties.—Because the surrounding medium may be an influencing factor, the studies were made using distilled water, 2% sodium chloride, 0.5% hydrochloric acid, 1% sodium bicarbonate, artificial gastric juice (15) and artificial intestinal juice (16). Five grams of each product was placed in a graduated cylinder and the volume of the product measured. For tablet volume, the tablets were placed in a graduated cylinder, a definite volume of water added and the volume occupied by the tablets noted. Each product was then placed in a 500-cc. graduated cylinder, sufficient liquid added at 22° to bring the volume to 50 cc. and the suspension mixed for fifteen seconds by means of a long glass stirring rod. More liquid was added to bring the volume to 200 cc. and the suspension mixed again for fifteen seconds. The volume was then brought to 500 cc. by carefully adding more of the liquid. The liquid was added by means of a long piece of glass tubing bent up at the end to form a "u," and the opening at the "u" was held at the surface of the liquid during its addition. This minimized agitation and further mixing of the 200-cc. bulk-laxative suspension. The cylinders were allowed to stand at room temperature and the volumes of the bulk-laxative masses noted over a period of twenty-four hours.

Gel Textures.—The textures or consistencies of the bulk-laxative masses were observed by various means depending upon the type of mass formed. Masses made up of large gelatinous particles were observed and compared as they were stirred by means of glass rods, noting the ease of resuspension in the supernatant liquid, particle size, and sedimentation rate. The supernatant liquid was decanted off the granular, mucilaginous type of mass and observations made by stirring with a glass rod, rubbing between the fingers, and taking the viscosity at 22° by means of a Brookfield Viscometer, model RVF, 10 r. p. m. and spindle No. 6. Jelly-like, rubbery masses were observed by placing them on flat glass plates and noting their ability to quiver when shaken gently. Their rigidity, elasticity, and resistance to being torn apart by means of a glass rod were also compared. Some of the bulk laxatives formed gummy masses which were observed by decanting off the supernatant liquid, rubbing the masses

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† This paper is abstracted from a dissertation presented to the Graduate School of the University of Washington by Jack N. Bone in partial fulfillment of the requirements for the degree of Doctor of Philosophy.
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between the fingers, and taking their viscosities at 22° by means of a Brookfield Viscometer, model VF, 20 r. p. m. and spindle No 7.

EXPERIMENTAL

Twenty-four bulk laxatives were compared as to their swelling properties and gel textures by the methods outlined above. Those studied are designated in the following list by composition or bulk laxative group rather than by trade names. The form of the product is designated by the words in parentheses.

1. Carob, 50%; acacia, 50% (powder)
2. Carob (granules)
3. Methylcellulose (tablets)
4. Methylcellulose, 9% (liquid)
5. Methylcellulose, 75%; psyllium, 25% (tablets)
6. Methylcellulose, 86%; prune concentrate, 14% (tablets)
7. Methylcellulose, 75%; prune concentrate, 25% (tablets)
8. Tragacanth (granules)
9. Tragacanth, 87%; magnesium trisilicate, 8.7% (granules)
10. Tragacanth (granules)
11. Tragacanth, 95%; kaolin, 5% (granules)
12. Tragacanth (granules)
13. Psyllium, 80%; prune powder, 20% (granules)
14. Psyllium (granules)
15. Psyllium, 50%; dextrose, 50% (granules)
16. Psyllium (large flakes)
17. Psyllium (powder)
18. Psyllium, 50%; lactose, 37.5%; dextrose, 12.5% (powder)
19. Psyllium, 50%; dextrose, 50% (fine flakes)
20. Psyllium, 50%; dextrose, 50% (fine flakes)
21. Psyllium (mixture heavy and light large flakes)
22. Psyllium, 50%; malt extract, 50% (powder)
23. Psyllium; mineral oil (emulsion)
24. Psyllium; agar; mineral oil (emulsion)

The comparative data collected are shown in Tables I to IV. Table I shows the swelling properties of the bulk laxatives in distilled water and shows the general trend of swelling according to time. Tables II, III, and IV show the swelling properties of the bulk laxatives in the various liquid suspending media at the end of one, four, and twenty-four hours.

DISCUSSION

Swelling Properties.—For preparations in the form of granules or flakes which suspend as the liquid is added and then settle to the bottom, this procedure seems satisfactory in making comparative studies of the swelling properties of various bulk laxatives. It does not, however, show the increase in swelling according to time for powdered preparations. Their largest mass volumes are produced at the beginning of the observation period then gradually diminish. For tablet preparations there is a strong tendency for the tablets to stick firmly together on the bottom of the graduated cylinder. If they do break loose they float to the top, then, upon disintegration shed particles which settle (or sometimes float) and this greatly influences the volume occupied by the preparation. This procedure gives no information on products which are readily miscible with the suspending liquid. Samples No. 10 and No. 11 are quite acid and these liberated CO_2 from the $NaHCO_3$ solutions. This liberated gas agitated the mass during the first part of the observation period.

The swelling properties of the carob group of bulk laxatives were not influenced to any extent by the different types of suspending liquids used. Their volumes increased from 5 to 10 times in twenty-four hours with most of the swelling taking place during the first hour. The methylcellulose tablets showed

TABLE I.—VOLUME IN MILLILITERS OF THE BULK-LAXATIVE MASS IN DISTILLED WATER

Sample Number	Initial Volume	Time in Hours					
		1	2	3	4	5	24
1	8	250	250	240	230	220	60
2	9	40	40	40	40	45	45
3	5	5	5	15	25	25	25
4	5	*[a]	*	*	*	*	*
5	5	5	5	7	7	7	35
6	5	5	10	35	40	40	55
7	5	5	5	10	10	25	35
8	8	175	250	280	300	310	355
9	8	125	150	155	160	160	160
10	7	210	250	275	280	285	305
11	6	125	140	145	145	145	150
12	9	180	230	255	275	285	360
13	8	50	60	60	60	60	60
14	10	55	70	75	75	75	80
15	12	60	60	60	60	60	60
16	13	60	60	60	60	60	60
17	9	235	225	220	220	215	175
18	9	200	190	190	190	190	190
19	7	140	140	135	130	130	110
20	8	160	155	150	145	145	115
21	15	375	375	350	320	315	245
22	9	95	90	85	85	85	75
23	5	*	*	*	*	*	*
24	5	*	*	*	*	*	*

[a] *—Miscible with the suspending liquids.

TABLE II.—NUMBER OF TIMES INCREASE IN VOLUME OVER INITIAL VOLUME OF BULK LAXATIVES IN ONE HOUR

Sample Number	Suspending Liquid: Dist. H_2O	2% NaCl	0.5% HCl	1% $NaHCO_3$	Artificial Gastric Juice	Artificial Intestinal Juice
1	31	22	16	21	22	22
2	4	5	5	4	5	4
3	0	0	0	0	0	0
4	*[a]	*	*	*	*	*
5	0	0	1	1	0	0
6	0	0	2	2	2	2
7	0	0	1	1	2	2
8	22	8	8	11	8	13
9	16	9	9	11	9	11
10	30	11	11	16	11	18
11	21	12	11	18	13	20
12	20	10	10	13	10	15
13	6	8	7	8	7	7
14	6	5	5	6	5	6
15	5	5	6	5	5	5
16	5	5	5	4	5	5
17	26	12	12	15	13	15
18	22	13	13	18	16	17
19	20	9	9	8	9	8
20	20	7	6	6	7	6
21	25	11	8	8	10	8
22	11	7	6	8	6	7
23	*	*	*	*	*	*
24	*	*	*	*	*	*

[a] *—Miscible with suspending liquids.

the least disintegration and increase in swelling in the distilled water, 5–11 times in twenty-four hours, but, in the other suspending liquids used, the tablets disintegrated much better and as a result swelled from 12 to 30 times in the NaCl and alkaline solutions and 6–18 times in the acid solutions. The methylcellulose tablet preparations swelled gradually over the twenty-four hour period.

With the tragacanth group the swelling was influenced by the type of suspending liquid used. The most swelling occurred in the distilled water, from 20 to 44 times the original volume in twenty-four hours. The mass volumes were about one-half as great in the $NaHCO_3$ solution and in the artificial intestinal juice as in the distilled water. They were still smaller in the NaCl solution, the HCl solution

TABLE III.—NUMBER OF TIMES INCREASE IN VOLUME OVER INITIAL VOLUME OF BULK LAXATIVES IN FOUR HOURS

Sample Number	Suspending Liquid: Dist. H_2O	2% NaCl	0.5% HCl	1% $NaHCO_3$	Artificial Gastric Juice	Artificial Intestinal Juice
1	30	21	12	21	22	19
2	4	5	6	5	6	4
3	0	12	3	4	5	8
4	*[a]	*	*	*	*	*
5	1	6	3	7	5	5
6	8	12	8	11	9	14
7	2	9	6	8	7	12
8	38	13	11	18	11	16
9	20	12	11	14	12	14
10	40	16	15	22	15	22
11	24	16	16	24	16	23
12	31	16	15	18	16	21
13	8	8	7	8	7	7
14	8	6	7	7	6	7
15	5	7	6	5	6	5
16	5	5	5	5	6	5
17	25	12	12	14	13	4
18	21	13	13	16	15	15
19	19	9	9	9	9	8
20	18	8	6	7	7	6
21	21	11	9	9	10	8
22	10	6	6	8	6	7
23	*	*	*	*	*	*
24	*	*	*	*	*	*

[a] *—Miscible with the suspending liquids.

TABLE IV.—NUMBER OF TIMES INCREASE IN VOLUME OVER INITIAL VOLUME OF BULK LAXATIVES IN TWENTY-FOUR HOURS

Sample Number	Suspending Liquid: Dist. H_2O	2% NaCl	0.5% HCl	1% $NaHCO_3$	Artificial Gastric Juice	Artificial Intestinal Juice
1	8	7	10	9	8	10
2	5	6	7	5	6	5
3	5	30	6	16	6	30
4	•[a]	•	•	•	•	•
5	7	12	8	15	8	16
6	11	19	17	17	17	28
7	7	20	15	18	15	27
8	44	15	13	19	13	18
9	20	13	12	16	12	16
10	44	18	16	24	16	24
11	25	18	16	25	17	25
12	40	18	17	21	17	22
13	8	7	7	8	7	7
14	8	7	8	8	7	8
15	5	7	6	6	6	5
16	5	5	5	5	5	5
17	19	11	11	13	13	14
18	19	11	11	13	12	13
19	16	9	7	9	9	9
20	14	7	6	7	7	6
21	16	11	9	9	10	9
22	8	7	6	7	6	7
23	•	•	•	•	•	•
24	•	•	•	•	•	•

[a] •—Miscible with the suspending liquids.

and the artificial gastric juice. These preparations swelled gradually over the twenty-four hour period.

With the psyllium group the swelling was not influenced to any extent by the different types of suspending liquids used, except those in powdered and fine flake forms. These swelled more in distilled water than in the other liquids. The granular form swelled approximately 7 times in all the liquids. The powdered or fine flake forms swelled approximately 15 times in distilled water and 10 times in the other liquids. These preparations did most of their swelling during the first hour.

Gel-Textures.—The bulk laxative masses which were obtained at the end of twenty-four hours were divided into four classes: (*a*) Gelatinous particles, easily resuspended in the supernatant liquid when stirred with a glass rod. Members of the tragacanth group of bulk laxatives produced masses belonging to this class.

(*b*) Granular, mucilaginous masses. Members of the psyllium group in a powdered or fine flake form produced masses belonging to this class.

(*c*) Jelly-like rubbery masses. Such masses were produced by the psyllium group marketed in a granular or heavy large flake form.

(*d*) Gummy masses. The carob group and the methylcellulose group in tablet form produced masses belonging to this class.

The gelatinous particle class had the largest particles and the longest settling time in distilled water and the smallest particles and shortest settling time in the acid-sodium chloride solutions. In the alkaline solutions the particle sizes and settling times fell in between those of the distilled water and acid-sodium chloride solutions.

The viscosities of the granular, mucilaginous masses were greatest in the acid solutions, less in the sodium chloride and alkaline solutions, and least of all in the distilled water.

The textures of the jelly-like, rubbery masses were approximately the same in all the suspension liquids used.

The viscosities of the carob gummy masses were about the same in all the suspension liquids. The viscosities of the methylcellulose gummy masses varied greatly depending upon the disintegration of the tablets. These varied from masses so viscous as to be beyond the range of the viscometer (2,000,000 centipoises) to soft masses with a viscosity of 1400 centipoises.

SUMMARY

1. A study has been made on the swelling properties and gel textures of 24 commercially available bulk-type laxatives belonging to the psyllium, tragacanth, carob, and methylcellulose groups. These properties and textures were observed in distilled water, 2% sodium chloride, 0.5% hydrochloric acid, 1% sodium bicarbonate, artificial gastric juice, and artificial intestinal juice.

2. A procedure has been devised which produced results showing that: (*a*) The swelling of the psyllium group was not influenced to any extent by the different types of suspending liquids used but the form in which the product was marketed did influence the extent of swelling.

(*b*) The swelling of the tragacanth group was influenced by the type of suspending liquid, swelling the most in distilled water, less in the alkaline solutions, and least of all in the salt and acid solutions.

(*c*) The swelling of the carob group was not influenced to any extent by the different types of suspending liquids used.

(*d*) The swelling of the methylcellulose group in tablet form was the greatest in the sodium chloride and alkaline solutions. Less swelling was observed in the acid solutions and least of all in the distilled water.

3. In artificial intestinal juice the average number of times the bulk laxatives increased in volume in twenty-four hours were: the psyllium group, 5–14 times; the tragacanth group, 16–25 times; the carob group, 5–10 times; and the methylcellulose group, 16–30 times.

4. The procedure produced the following classes of bulk-laxative masses: large gelatinous particles; granular, mucilaginous masses; jelly-like, rubbery masses; and gummy masses.

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A Preliminary Phytochemical Investigation of *Digitalis orientalis**

By DAVID H. GREGG and OLE GISVOLD†

The cardiac glycosides of the fresh first-year leaves of *Digitalis orientalis* have been partially resolved into acetyl-digoxin, amorphous *digitalinum verum*, and other amorphous glycosides. A new and useful color reaction for paper chromatography of the digitalis cardiac glycosides has been developed. The periodate-benzidine reagent has been applied for detection and structural studies in this field. Some paper chromatographic studies have been reported.

IT HAS BEEN well established that in the *Digitalis* species, the age of the plant and its habitat determine the nature and amounts of the cardiac glycosides produced. In 1941, Mannich and Schneider (1), upon the investigation of *Digitalis orientalis*, found α- and β-acetyl digoxins and oridigin, a new glycoside, in small amounts together with the major amorphous glycosides which contained chiefly *digitalinum verum*. Because of our recent interest in the search for the acetyl cardiac glycosides and digitoxin, fresh leaves from the first year's growth of *Digitalis orientalis* were subjected to a preliminary phytochemical investigation. These studies have been aided in part by newer simplified enzyme-inhibiting techniques that have been previously published (2). The plants were grown in the Botanical Garden of the Department of Pharmacognosy from seeds that were obtained from A Stoll.

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EXPERIMENTAL

Primary Extract.—A primary aqueous extract of 20 Kg. of fresh (about 80% moisture) of *Digitalis orientalis*, grown in Minnesota in the summer of 1952, was prepared according to previously published procedures (2). Filtration of the coagulated disintegrated leaves was markedly simplified by using a fine wire 200-mesh Monel metal screen. No filter cell was necessary. An occasional cleaning of this screen was all that was required for smooth operation.

Ketone Extract.—Extraction of the aqueous extract with methyl isobutyl ketone (MIBK) yielded some clear MIBK extract plus some emulsified MIBK. The emulsion was partially cracked by centrifuging at 1600 r. p. m. for fifteen minutes. The residual emulsified material was cracked with the aid of anhydrous sodium sulfate. A dark brown solid interphase weighing 8.1 Gm. was obtained which, upon extraction with methanol, gave a small

amount of glycosidic material. Concentration of the MIBK under vacuum yielded 25.7 Gm. of a green semisolid which contained no crystalline material. This material was dispersed on filter cell with the aid of methanol and exhaustively extracted, successively, with petroleum ether, absolute ether, and ethanol. The extraction with ether gave a soluble and an insoluble fraction.

Ether-Insoluble Fraction.—This fraction was partitioned between 50% aqueous methanol and MIBK. All attempts to obtain crystalline material from the aqueous methanol phase failed. It was then combined with the ether-soluble fraction, because its insolubility in ether was believed to be only relative to the amount of solvent used. The MIBK fraction was decolorized and concentrated to a small volume. Several crops of pale tan spheres were obtained that weighed about 550 mg. Attempts to obtain this fraction in a crystalline form from various solvents and solvent mixtures gave indefinite results. The material appeared to be semicrystalline. It melted at 151–160°, gave a positive Keller-Kiliani test, with some red at the interphase, gave a negative periodate–benzidine test (*loc. cit.*) and gave a positive Gregg and Gisvold test (*loc. cit.*).

The MIBK mother liquors were combined with the ether-soluble fraction.

Ether-Soluble Fraction.—This fraction, 9.3 Gm., was subjected to chromatographic analysis using a column of aluminum oxide 11 cm. high that contained 60 Gm. The column was eluted and the various fractions collected in test tubes by means of an automatic fraction collector. The results of this analysis are presented in Table I.

periodate-benzidine test, and a positive Gregg and Gisvold test.

Ethanol-Soluble Fraction.—Upon concentration of this fraction 3.60 Gm. of a tan amorphous solid was obtained. It melted at 153–160° and gave a positive Keller-Kiliani reaction with some red at the interphase. This fraction was washed first with ether and then with chloroform. There resulted a chloroform-soluble and chloroform-insoluble fraction.

Chloroform-Soluble Fraction.—Concentration of the chloroform washings yielded about 260 mg. of a white amorphous solid. It melted at 158–168° and gave a positive Keller-Kiliani reaction with a tinge of red at the interphase. Crystallization from methanol followed by crystallization from chloroform, methanol, and ether yielded 115 mg. of crystalline rectangular platelets that, upon recrystallization, melted at 219–220°. The Keller-Kiliani test was positive. The periodate-benzidine test was negative and the Gregg and Gisvold test was positive.

Anal.—Calcd. for acetyl digoxin $C_{43}H_{66}O_{15}\cdot 1^1/_2CH_3OH$; C, 61.36; H, 8.33. Found: C, 61.42; H, 8.73.

The mother liquors (chloroform, methanol, and ether) upon spontaneous concentration yielded spheres of very short, needle-like crystals that melted at 161–167°, gave a positive Keller-Kiliani reaction, and a negative periodate-benzidine test.

The chloroform mother liquor from the 260 mg. of glycosidic materially yielded 1.13 Gm. of an amorphous mass of glycosidic material that could not be crystallized.

TABLE I.—CHROMATOGRAPHIC ANALYSIS OF THE ETHER-SOLUBLE FRACTION

Solvent	Volume, Ml.	Residue Weight, Gm.	Residue Description	Keller-Kiliani
Chloroform (Fraction 1)	200	4.72	Light green semisolid	Positive, red at interphase
Chloroform-ethanol (9:1) (Fraction 2)	100	2.06	Greenish tan semisolid	Positive, red at interphase
Fraction 2	300	0.79	Green semisolid	Negative
Fraction 2	100		Green semisolid	Negative
95% Ethanol	1,000	1.05	Orange solid	Negative
50% Ethanol	500	1.48	Orange solid	Negative
25% Ethanol	500		Red orange solid	Negative

When the residue weight in each tube was plotted on graph paper against the tube number, two peaks were formed: one large peak in the chloroform fraction, and a sharp smaller peak in the chloroform-ethanol fraction.

Fraction 1 resisted all attempts to obtain a crystalline material.

Fraction 2 was washed with petroleum ether. A chloroform, methanol, and ether solution of the petroleum ether-insoluble residue gave 100 mg. of an amorphous glycosidic material. After standing one month in the refrigerator, the mother liquor deposited a small amount of a crystalline glycoside that had a double melting point of 162–167°/200–212°. It gave a positive Keller-Kiliani test with a reddish-brown layer at the interphase, a negative

Chloroform-Insoluble Fraction.—This fraction, 1.85 Gm., was partitioned between 50% aqueous methanol and MIBK. Concentration of the aqueous phase yielded 850 mg. of a reddish-brown, hard, glossy, solid residue that resisted all attempts to obtain crystalline material. After washing with ether, this glycosidic material melted at 128–145°. It gave a positive Keller-Kiliani test with a bright red at the interphase. Acetylation (1) failed to yield a crystalline derivative even after it was chromatographed (3) on aluminum oxide. This glycosidic material may be *digitalinum verum*.

Concentration of the MIBK-phase under vacuum yielded a small amount, 30 mg., of a glycoside that, upon purification and analysis was shown to be acetyl-digoxin. The main MIBK-soluble material

weighed 860 mg. and resisted all attempts to obtain crystalline material. It melted at 157–185°, gave a positive Keller-Kiliani test with a red-brown at the interphase, and gave a positive Gregg and Gisvold reaction. The periodate-benzidine test was inconclusive.

Paper Chromatographic Studies.—Because paper chromatography has been utilized as a powerful tool in so many fields, considerable effort was expended in an attempt to find a solvent or solvent mixture that could be used not only for qualitative studies, but also for the separation of glycosides by means of a powdered paper column. The solvent mixture used by Svendsen and Jensen (4) did not prove satisfactory. When the solvent ratio for chloroform, methanol, and water was 10:4:5, the best results were obtained, but these were far from satisfactory. The results were not reproducible. The solvent front would not remain even, and the glycosides tested would either not move at all or they would streak or tail. These results were obtained despite the efforts to control the temperature. Therefore other solvents and solvent mixtures were tested. When aqueous MIBK was used, the glycosides followed the solvent front. A solvent mixture of methylchloroform, methanol, and water produced results similar to that of chloroform, methanol, and water. Digoxin gave an R_f value of 0.90 when chromatographed on Whatman No. 4 filter paper with a solvent mixture of *n*-butanol, methanol, and water in the ratio of 10:1:10. However, acetyldigoxin and digitoxin followed the solvent front with this solvent.

The following satisfactory R_f values for acetyldigoxin 0.88, digoxin 0.82, and Lanatoside-C 0.22 were obtained when the following conditions were used, i. e., (*a*) water saturated ethyl acetate, (*b*) equilibration time, four hours, (*c*) development time, six hours, (*d*) temperature 25°, and (*e*) Whatman No. 2 paper. However, digitoxin and acetyldigitoxin gave R_f values too close to that of acetyldigoxin.

Also, the following satisfactory R_f values for acetyl-digoxin, 0.88, digoxin, 0.75, and Lanatoside C 0.049 were obtained when the solvent mixture was MIBK, MEK (methyl ethyl ketone) and water in the ratio of 10:2:5. The temperature was 25°, the development time was three hours, the equilibration time was sixteen hours, and Whatman No. 2 paper was used.

A constant boiling mixture of MEK and water gave results similar to those described above. It had the advantage that no equilibration period was necessary. However, as in the preceding experiment, no separation on paper could be obtained with mixtures of acetyl-digoxin, acetyl-digitoxin, and digitoxin.

Ascending Paper Chromatography.—This technique has some advantages over the descending techniques, i. e., simplicity of apparatus and rapidity of development. This permits the rapid screening of a large number of solvents in a minimum amount of time. Sheets of Whatman No. 2 paper were cut into strips 12 × 5 cm. They were rolled into a half cylinder and placed in a 250-cc. Erlenmeyer flask with a small beaker for a cover. The compounds to be chromatographed were applied to the paper on a line drawn 18 mm. from the short side. The development time was usually eight minutes. The Gregg and Gisvold reagent was used to detect the glycosides. When the solvent mixture used was benzene, methanol, and water (100:30:1) the following R_f values were obtained, Lanatoside C, 0.27, digoxin, 0.46, acetyl digoxin, 0.72.

Color Reactions.—Although a number of color reactions are reported for the detection of the digitalis cardiac glycosides on paper, none are simple and easy to use. The use of trichloroacetic acid in the test of Svendsen and Jensen (4) had a number of disadvantages, i. e., (*a*) the paper often became charred during the heating period, (*b*) a darkroom with an ultraviolet lamp was needed, (*c*) the fluorescent spots were sometimes difficult to read because of the fluorescent impurities in the paper and (*d*) it does not detect the presence of digitoxose. On the other hand, it is sensitive to one gamma of the glycoside and will detect the genin.

In a search for a sensitive satisfactory color test for the detection of some of the digitalis cardiac glycosides, the following experiment was performed. Digitoxin dried on Whatman No. 2 paper, was sprayed with 1% hydrogen chloride in methanol. The paper was heated in an electric oven at 140° for one minute. A gray spot which rapidly became a distinct blue-gray in color was observed on the paper. The acetyl-digitoxins, acetyl-digoxins, digoxin, gitoxin, Lanatoside C, and gitalin amorphous gave the same test. Because ferric chloride increases the sensitivity of the Keller-Kiliani reaction, one half cc. of a 10% solution of ferric chloride was added to 100 cc. of the 1% hydrogen chloride in methanol (2.8 cc. conc. HCl and 97.2 cc. distilled water). The addition of the ferric chloride did not increase the sensitivity of the reagent and the test, but the spots appeared to be bluer and were more readily detected on a yellow background. Because some of the sheets became charred when heated in the oven and to simplify the source of heat if possible, it was shown after some experimentation that one or more 250-watt infrared lamps were a satisfactory source of heat. A standard heating time of seven minutes at a lamp distance of 30 cm. from four lamps mounted one above the other was adopted to give no charring. The filter papers were mounted on a heavy asbestos board. The reagent for convenience will be referred to as the Gregg and Gisvold reagent and the test as the Gregg and Gisvold test.

The Gregg and Gisvold test when applied to digitoxose isolated from the hydrolysis (5) of crude acetyl-digoxin gave a positive blue-gray. Crystalline digoxigenin did not give a positive blue-gray color spot. Therefore, this test detects the digitoxose portion of the digitalis cardiac glycosides. This test is not specific for digitoxose, because sucrose, fructose, and sorbose also give the same test. Glucose gave no color whereas D-arabinose gave a yellow-green color; D-galactose and L-ascorbic acid each gave a pink color. It is interesting to note that the mother liquor from the crystalline digitoxose when examined by paper chromatography using Whatman No. 1 paper and MEK-water azeotrope as a solvent gave gray-blue spots with R_f values of 0.47, 0.69, 0.75, and 0.80. These spots did not fluoresce and therefore no genin was present.

Because the trichloroacetic acid used in the Svendsen and Jensen (4) fluorescent test is a strong acid, there was the possibility that the substitution of other strong acids might give the same results.

This postulation was verified when it was shown that the blue-gray spots of the Gregg and Gisvold test showed a blue fluorescence when examined under the ultraviolet light thirty minutes after heating. Therefore, this test can serve to detect both the digitoxose and the genin portion of the digitalis cardiac glycosides.

Sensitivity of the Gregg and Gisvold Test.—This test, when applied to various quantities of acetyl-digoxin, showed fluorescence under the ultraviolet lamp to quantities as low as 0.63 gamma but not to 0.31 gamma. Equal sensitivity was exhibited by other digitalis cardiac glycosides. The glycosides were applied to Whatman No. 2 paper with a calibrated wire loop that deposited 0.0037 cc.

Position of the Acetyl Group in the Digitalis Cardiac Glycosides.—The development of the periodate-benzidine test[1] enables the rapid detection of *cis* 1,2-glycols on paper. The substance to be tested on paper is first sprayed with a saturated aqueous solution of potassium periodate. After one minute, the paper is sprayed with a solution of the following composition: 10 parts of 0.1 *M* benzidine dissolved in a mixture of 80 parts of ethanol and 20 parts of acetone and 1 part of 0.2 *N* hydrochloric acid. A positive test is shown by a colorless spot. Digoxin, digitoxin, and gitoxin gave a positive test whereas α- and β-acetyl-digoxin and α- and β-acetyl-digitoxin gave a negative test. It can be concluded that one can differentiate on paper between the acetyl-digoxins and digoxin and the acetyl-digitoxins and digitoxin. It also can be concluded that these simple tests prove that the acetyl group in the acetyl-digoxins and acetyl-digitoxins must be present on the terminal digitoxose residue. This digitoxose residue is the only portion of these compounds that can contain a *cis* glycol structure if it is present as the pyranose modification. A pyranose modification must be present because a positive periodate-benzidine test is obtained with digoxin, digitoxin, and gitoxin.

Lanatosides A and C also fail to give a positive periodate-benzidine test. In addition to the above conclusions, these results indicate that the terminal glucose residue in these glycosides contains a pyranose ring. This conclusion is based upon the fact that the pyranoside modification of the glucose residue contains no *cis* glycol structure. Under similar test conditions, methyl α-D-glycopyranoside fails to give a positive test. Furthermore monoacetone glucose in which a furanose structure is known to be present and in which the hydroxyl groups on carbon atoms 5 and 6 are not substituted gives a positive test under similar test conditions.

The above evidence serves to substantiate A. Stoll's (6) postulates that the acetyl group in the glycosides is on the third digitoxose residue.

[1] Private communication with F. Smith, University of Minnesota.

SUMMARY

1. The presence of small amounts of acetyl-digoxin in the fresh first-year leaves of *Digitalis orientalis* grown in Minnesota has been established.

2. The chief glycosides of *Digitalis orientalis* are amorphous and resemble *digitalinum verum*.

3. Several new solvent mixtures were developed for the successful separation of digoxin, acetyl-digoxin, and Lanatoside C, on both descending and ascending paper chromatography.

4. The R_f values for digitoxin, acetyl-digitoxin, and acetyl-digoxin were so close to each other with the solvent mixtures studied that no satisfactory separation could be effected.

5. A new simple color test has been developed to detect those digitalis cardiac glycosides containing digitoxose. The genin also can be detected by the fluorescence under an ultraviolet lamp in the dark. This test has been designated as the Gregg and Gisvold test.

6. The sensitivity to the fluorescent reaction for the Gregg and Gisvold test has been shown to be about 0.60 gamma for the digitalis cardiac glycosides.

7. The periodate-benzidine test has been used to establish the position of the acetyl group in the digitalis cardiac glycosides as being on the terminal digitoxose residue.

8. The periodate-benzidine test has been used to establish the ring structure as pyranose in the glucose residue of Lanatosides A and C and in the terminal digitoxose residue of digoxin, digitoxin, and gitoxin.

9. The periodate-benzidine test when teamed with the Gregg and Gisvold test can be utilized to differentiate between acetyl-digoxin and digoxin and between acetyl-digitoxin and digitoxin.

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Heparinic Effects of Cupridihydroporphyrins (Water-Soluble Chlorophyllins)*

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The medicinal, water-soluble chlorophyllin Chloresium and some of its derivatives are shown to be heparin-like substances in that they act as an antithrombic in the blood coagulation mechanism and antagonize at least one mucolytic system as exemplified by glycoprotein and type-specific hemagglutination. The literature indicates that they may likewise share the heparin effect of altering the lipoprotein pattern in atheromatosis. If this were confirmed, complete parallelism between heparinoids and metalloporphyrins would be established.

IN A PREVIOUS STUDY, a distinct antithrombic action of lithium ferrihemate was reported (1). The observation, as well as the substance eliciting it were considered of only academic interest, the effect itself of a much lower order than that exhibited by the classic antithrombic, heparin. Lithium ferrihemate had only been incidentally prepared to obtain a ferriprotoporphyrin which would be water-soluble at physiologic *p*H and devoid of the surface activity (hemolytic properties) inherent in the other alkali metal salts of the pigment. Since the original observation, the antithrombin heparin has seen its physiologic functions broadened beyond those of a purely anticoagulant substance; a role has been ascribed to it in the regulation of thrombocyte output (2), as the physiologic hyaluronidase inhibitor (3), and as the mediator of chylomicron dispersion (4).

A revival of interest in the antithrombic effect of ferriheme resulted from a more recent observation. During the course of intravenous administration of Chloresium (sodium cuprichlorophyllin) solutions to patients, it was noted that the blood refluxed into the syringe remained fluid inordinately long; where the residual amount of Chloresium in the syringe was sufficient, this prolongation of the clotting time was infinite. The nature of this effect was studied and proved to be antithrombic. This similarity of anticoagulant effects between chlorophyllin and heparin led to a scrutiny of other possible shared effects such as antimucolytic (antihyaluronidase), and lipotropic effects.

EXPERIMENTAL

Anticoagulant Effects.—Human venous blood was aspirated through a 20-gauge needle into a 10-cc. saline-moistened glass syringe and 2-cc. aliquots rapidly ejected into vacutainers made up with varying amounts of either dibasic or tribasic potassium chlorophyllinate in 4% solution to yield final chlorophyllinate concentrations in the individual blood samples ranging from 0.0125 to 0.2%. At various intervals, the rack containing the tubes was tilted to determine the inception of coagulation as in the Lee-White method (5) for ascertaining this end point of the coagulation reaction. Seven determinations were made of the dibasic and three of the tribasic chlorophyllin effect, all on patients with eucoagulability status.

The results with dibasic potassium cuprichlorophyllinate are shown in Table I which represents a typical experiment. Similar results, all indicating that the delay in coagulation is a discontinuous exponential function of the concentration, have been obtained with the tribasic cuprichlorophyllin (cuprichlorin e_4), the dibasic cobaltichlorophyllin, and with an antibacterial, homogeneous fraction from the commercial water-soluble chlorophyll derivatives—sodium cupri-isochlorin e_4. In all instances, the blood rendered incoagulable by these agents did not clot on addition of calcium, and all showed a dissociation of filar and gelatinous fibrin precipitation on additions of subliminal amounts of the pigment. These features are all indicative of antithrombic activity (2), and it seems likely that this activity is a common property characteristic of the transition element metalloporphyrins.

Because of the current wide medical utility of the metallodihydroporphyrins (chlorophyllins) as well as the broadened implications of heparinic activity, further studies with the effect of water-soluble chlorophyllin on mucolytic enzyme systems and chylomicron distribution appeared to be in order.

Mucolytic Systems.—Wattenberg and Glick (6) have shown that ferriheme is a marked hyaluronidase inhibitor. Removal of the iron to yield protoporphyrin reduced this activity, while removal of vinyl groups (as in coproporphyrin or stercobilin) abolishes it altogether. Glick's group ad-

TABLE I.—ANTICOAGULANT EFFECTS OF CHLOROPHYLLINATE CONCENTRATIONS

Coagulation Time, Min.	Concentration K_3 Chlorophyllin, %
6.5	0.0125
10.5	0.025
35.0	0.05
480.(?)	0.1
Infinity	0.2

* Received August 10, 1953, from the Chemistry Section, Laboratory Division of the Research Department, Rystan Company, Inc., Mount Vernon, N. Y.

duced strong evidence that heparin was probably the antihyaluronidase operative during physiologic stress, but could not prove this conclusively because of the competitive inhibition in the hyaluronidase-hyaluronic acid system, as heparin is chemically similar to the substrate. Bergamini and Ferrari (7) showed lysozyme to be inhibited by heparin and related anticoagulants, polysulfonates; Kerby and Eadie (8) proved heparin inhibition of lysozyme to be competitive using *M. lysodeikticus* as the substrate.

Preliminary experiments designed to demonstrate the heparin-like effects on mucolytic enzymes indicated that Wattenberg and Glick's elucidation of the antihyaluronidase effect of vinyl group-containing porphyrins (3) is carried over to the chlorophyllins (which invariably contain one such group). In screening experiments, human semen specimens submitted for laboratory fertility evaluation were blended with 0.2% Chloresium solution to a final pigment content of 0.02%. Results with "normal" semen were equivocal in that no inhibition of spontaneous liquefaction was observed, but because of the usual delay in the receipt of such specimens, their very high hyaluronidase content, and the possibility that partial depolymerization of hyaluronidase may have occurred before the admixture of chlorophyllin, led to studies with relatively infertile specimens. There appears to be a significant correlation between the sperm and the hyaluronidase content of semen and in oligospermic specimens, which liquefied at all, this liquefaction could be entirely prevented by 0.002% chlorophyllin content. The degree of mucolytic enzyme inhibition could not be quantitatively established in this system, but qualitatively it was quite definite.

Experiments with purified hyaluronidase-sodium hyaluronate mixtures were therefore conducted. Additions were made to a buffered sodium hyaluronidase solution of a testes hyaluronidase, standardized by the turbidometric method of Dorfman and Ott (9). (This method of actual assay in the present system was precluded by the strong tint of chlorophyllin imparted to the reaction mixture.) Table II shows that cupri-isochlorin e_4, even in 1 part per 100,000, appreciably inhibited the viscosity drop of the polymer. However, again, this is of only qualitative significance because the viscosimetric methods in this system are notoriously affected by electrolyte content and there is no knowledge of the actual ionic strength contribution of the incorporated chlorophyllin derivative. For quantitative analysis, therefore, a biologic system was resorted to, comparable to that studied by Kerby and Eadie (8).

TABLE II.—INHIBITION OF VISCOSITY BY CUPRI-ISOCHLORIN e_4

Hyaluronidase[a] (T. R. U. Units)	Cupri-isochlorin e_4, %	Time (After Admixture)	Viscosity Ostwald
0	0	5	2.39
0	0	30	2.47
5	0	5	1.83
5	0	30	1.37
25	0	5	1.66
25	0	30	1.27
25	0.01	5	2.24
25	0.01	30	2.19
25	0.001	30	1.94
25	0.001	90	2.06

[a] Sodium hyaluronidase, 0.2%, in 7.8 isomolar phosphate buffer at room temperature (19°).

The Kerby-Eadie type of cellular system studied was that utilizing erythrocyte agglutination (instead of mycobacteriolysis) by mucinoid substances. The reaction of the human erythrocyte is a sensitive and quantitatively definable indicator of mucinoid depolymerization. Any depolymerized mucinoid will act as a hemagglutinating "blocking-type antibody" against erythrocytes when incorporated in a system which contains even subliminal traces of agglutinins for such cells. The various "conglutinin" techniques for eliciting Rh incompatibility in low titer of otherwise undemonstrable forms is based on this property. Given a serum containing anti-Rh agglutinins and Rh-positive cells which are coated so that they fail to agglutinate when exposed to such a serum, agglutination at once becomes evident if a low-molecular weight mucinous colloid, such as carboxymethyl cellulose, dextran, polyvinyl pyrrolidine, or hog gastric mucin is added to the system. The function of these conglutinins (something that fortifies or works along with a true agglutinin) seems to be an alteration in the dielectric constant of the solution since other low-molecular colloids—e. g., bovine serum albumen, modified human globin, and trypsinized gelatin—have all found utility in blood compatibility testing techniques where it is desired to eliminate the possibility of introducing an aberrant or low titer agglutinin or activating it in the circulation of the transfusion recipient.

High polymers, such as high molecular weight dextran, do not exhibit this conglutination effect and can be safely incorporated into transfused blood (10). Some of the low-molecular weight dextrans presently used for infusion are dangerous in this regard and have caused serious transfusion reactions (11). Similarly, sodium hyaluronate is not a conglutinin, but its mucolytic depolymerization product is a conglutinin and will cause spontaneous agglutination in many red-cell suspension specimens. This occurs where the cells are suspended in their own plasma, since normal plasma contains small (but subliminal) concentrations of a mucoprotein autohemagglutinin, and the effect is particularly pronounced in the blood of patients with certain neoplastic diseases, other adaptation diseases, or those who have received multiple transfusions—in which cases this mucoprotein fraction is markedly increased.

Hemagglutination titers in various sytems in the presence and absence of water-soluble dihydroporphyrins have been obtained. Data are presented in some detail in Table III. The incorporation of water-soluble chlorophyll derivatives markedly inhibits the hemagglutination produced from the depolymerized hyaluronate through hyaluronidase activity, but these derivatives also inhibit all types of hemagglutination *per se*.[1]

[1] Subsequent experiments have shown that all heme and chlorophyllin derived porphyrins, even those which are metal-free, are universal haptenes for hemagglutination systems, inhibiting not only the conglutinin effect but type specific and Rh agglutination as well (evident from data in Table III) (11). More remarkably, this haptene effect is carried over to the abrogation of cellular attachment by the hemagglutinating viruses (influenza *A* and Newcastle Disease) to the hemagglutinating phytotoxins (scarlet-runner bean and ricin) and to bacterial mucoproteins (*Hemophilus*).

TABLE III.—HEMAGGLUTINATION TITERS

System (Cell type–agglutinin)	Porphyrin	Per Cent	Hemagglutination Titer 1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256
D (Rh_0)–Anti D agglutinating	K Cu chlorophyllin	0.0	+[a]	+	+	+	−[b]	−	−	−	−
.....	.....	0.47	−	−	−	−	−	−	−	−	−
.....	.....	0.008	+	+	−	−	−	−	−	−	−
.....	.....	0.0016	+	+	+	−	−	−	−	−	−
.....	K pheophyllin	0.07	−	−	−	−	−	−	−	−	−
.....	K Co chlorophyllin	0.05	−	−	−	−	−	−	−	−	−
.....	.....	0.01	w[c]	vw[d]		−	−	−	−	−	−
.....	.....	0.002	+	w	vw	−	−	−	−	−	−
.....	K Cu chlorin e_3	0.006	−	−	−	−	−	−	−	−	−
.....	Na cuprisochlorin e_4	0.03	+	−	−	−	−	−	−	−	−
.....	.....	0.003	+	+	−	−	−	−	−	−	−
.....	.....	0.0003	+	+	+	−	−	−	−	−	−
D (Rh_0)–Anti D "blocking"	.....	0.0	+	+	+	+	+	+	+	+	+
.....	.....	0.03	+	+	−	−	−	−	−	−	−
.....	.....	0.003	+	+	w	−	−	−	−	−	−
.....	.....	0.0003	+	+	+	+	+	vw	−	−	−
B–Anti B	.....	0.0	+	+	+	+	+	+	−	−	−
.....	.....	0.03	+	+	−	−	−	−	−	−	−
.....	.....	0.0003	+	+	+	−	−	−	−	−	−
.....	K Cu chlorophyllin	0.08	+	−	−	−	−	−	−	−	−
.....	.....	0.016	+	+	−	−	−	−	−	−	−
.....	K Co chlorophyllin	0.003	+	+	+	+	−	−	−	−	−
A–Anti A	.....	0.00016	+	+	+	+	w	−	−	−	−
.....	.....	0.0	+	+	+	+	+	−	−	−	−
.....	.....	0.08	+	−	−	−	−	−	−	−	−
.....	.....	0.016	+	+	−	−	−	−	−	−	−
D–hyaluronidase (2 TRU)	.....	0.0	−	−	−	−	−	−	−	−	−
D–Na hyaluronate (0.2%)	.....	0.0	+	vw	−	−	−	−	−	−	−
.....	K cuprisochlorin e_4	0.003	−	−	−	−	−	−	−	−	−
D–hydase-uronate	.....	0.0	+	+	+	+	+	+	+	+	−
.....	.....	0.03	−	−	−	−	−	−	−	−	−
.....	.....	0.003	+	−	−	−	−	−	−	−	−
.....	.....	0.0003	+	+	−	−	−	−	−	−	−
.....	K Na ferrichlorin e_3	0.03	+	−	−	−	−	−	−	−	−
.....	K pheophyllin	0.3	+	+	−	−	−	−	−	−	−

[a] + —positive.
[b] − —negative.
[c] w—weak.
[d] vw—very weak.

The indicator of inhibition of the pertinent system was therefore affected by the purported inhibitor and in the same direction, so that it does not qualitatively demonstrate the chlorophyllin inhibition of mucolytic activity. It does quantitatively, however, when the relative inhibitions by comparable concentrations of isochlorin e_4 against the Rh blocking antibody and the depolymerized hyaluronate are considered. These agglutinations fortuitously had about the same titers; the reduction of the latter by isochlorin e_4 is significantly greater than that of the former. It can only be concluded that while part of the titer reduction by the isochlorin e_4 is due to the direct inhibition of hemagglutination, a sensible amount is based on the failure of the hyaluronate to depolymerize in the presence of the chlorophyllin derivative. Table III also shows that hyaluronidase by itself is neither directly hemagglutinative, nor does it inhibit agglutination in the presence of the depolymerized hyaluronate (*viz.*, when chlorophyllin is absent); the slight hemagglutination (1:2) of 0.2% unaltered hyaluronate is undoubtedly due to a small content of the depolymerized fraction present in all available preparations. This quantitative analysis, taken in conjunction with the qualitative semen and viscosimetric studies, leaves little doubt that Wattenberg and Glick's assignment of hyaluronidase inhibitory activity to vinyl-containing porphyrins can be extended to the medicinal water-soluble chlorophyllins.

The consistent antimucolytic and antihyaluronidase action of water-soluble chlorophyllins, as herein demonstrated, predicates for these substances the properties to which the anti-inflammatory effects of

locally applied cortisone and hydrocortisone are ordinarily attributed (12), thus furnishing an explanation for and rationalizing the entrenched clinical experience that water-soluble chlorophyllins have such an anti-inflammatory activity in wounds and on engorged mucous membranes. The demonstrated antihemagglutinative activity of water-soluble chlorophyllins, besides possible repercussions on blood transfusion practice, may serve to explain the rapid proliferation of (vascularized) granulation tissue from hitherto sluggishly healing or nonhealing wounds after the application of chlorophyllins—another fact repeatedly documented by experience but heretofore without complete explanation. The direct antihyaluronidase effect found operative furnishes a reasonable explanation of the "disinfection" of wounds by chlorophyllin and in the absence of marked bactericidal action of the latter, since the bacterial invasion of some species (pneumonococci, hemolytic streptococci, and clostridia) is reportedly implemented by their own hyaluronidase production. Furthermore, the "antivenom" effect, reported many years ago by Ribeiro and Guimaraes (13) for chlorophyllin, is believable because snake venoms owe their diffusion properties to their content of hyaluronidase.

Lipotropic Effects.—In a restricted sense, the term, lipotropism, has been applied to the mobilization of fat by the liver—a presumably salutory phenomenon that facilitates the detoxifying functions of that organ. While McCloskey and McGehee (14) have found intravenous chlorophylline to forestall carbon tetrachloride-induced hepatopathy in the rabbit (and we have seen resuscitation from hepatic coma in humans after intravenous administration of 3 mg./Kg. of Chloresium), no evidence linking this with heparinic effect is presently available. Lipotropism has recently been assigned a more general significance where heparin does participate—in the distribution of lipoprotein aggregation in plasma. An improper chylomicron distribution is supposedly conducive to atheromatous change in the human. Boyle, *et al.* (15), have used sequestrin, an antithrombic chelating agent, in the treatment of rabbit atherosclerosis.

Engenberg and Massell (16) have obtained good clinical results from intermittent intravenous heparin administration in human arteriosclerosis. They attribute this result to atherosclerosis inhibition through the demonstrated effect of heparin in altering the "lipoprotein spectrum of the blood." It should be pointed out in this connection that water-soluble chlorophyllins have had wide application in the treatment of human atherosclerosis (17), and that Gordonoff (18) demonstrated experimentally that rabbits on high cholesterol intake did not develop atherosclerosis if chlorophyllin was simultaneously administered. It is tempting to ascribe Gordonoff's results, as well as those reported clinically to the "heparin-like effect" of chlorophyllins, but this effect needs to be verified by further direct observation of the effect of chlorophyllin on chylomicron distribution. Such a study is in order because it is apparent from the demonstrated antithrombic and antihyaluronidase of these pigments that they cannot be considered physiologically inert; the extensive but neglected literature around the pharmacology of chlorophyllins merits renewed scrutiny.[3]

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[3] Since this paper was written, three additional similarities between heparin and metalloporphyrins have emerged. Cluff and Berthong [*Bull. Johns Hopkins Hosp.*, **92**, 353 (1953)] reported isoagglutinin titer diminution by heparin. This has been confirmed and extended to the demonstration of competitive inhibition by either heparin or cuprichlorophyllin of vaccinia virus hemagglutination and infectivity by the technique of McCrea and Duran-Reynolds [*Science*, **118**, 93(1953)], who curtailed vaccinia infectivity in rabbits with various hexuronic acids. When this was confirmed with either heparin (a polymer of hexuronic acid) or various chlorophyllins, an actual *chemical* conformity between the two apparently dissimilar haptene-like substances became obvious. Both contained resonating allelopentacyclic rings—furanose in the case of heparin and pyrrol in the case of chlorophyllin. Absence of the contributory nature of the nonresonating isopentacyclic (carbocyclic ring) in the dibasic chlorophyllins was then shown by comparative titration against tribasic chlorin e_3, which was found to have statistically greater effect in all of the parameters; it has antihemagglutinative, antihyaluronidase, antiviral (with present restriction to the hemagglutinating viruses), and antithrombic activity. It was tentatively decided that these haptene effects could be anticipated as a generic property of any resonating pentacyclic ring isosteric with the furanose of mucoproteins or polysaccharides when Tamm, Folkers, Shunk, Heyl, and Horsfall [*J. Exptl. Med.*, **98**, 245(1953)] reported the inhibition of influenza virus infectivity and hemagglutination by the benziminazoles. The latter have such an allelopentacyclic ring isosteric with furanose. The implications of the incidental elucidation of determinant chemical configurations, indicating the possible nature of competitive hyaluronidase inhibition and the configurations of cellular receptors to which hemagglutinins, viral and otherwise, attach, will be discussed in a definitive report.

Effects of Liver Damage and Nephrectomy on Anticonvulsant Activity of Mysoline and Phenobarbital*

By EWART A. SWINYARD, DAVID H. TEDESCHI, and LOUIS S. GOODMAN

Mysoline and phenobarbital were tested after their oral administration in normal, liver-damaged, and nephrectomized rats for their ability to prevent the tonic extensor component of maximal electroshock seizures. The results were analyzed for the effect of these procedures on the intensity and the duration of anticonvulsant action of these compounds. Liver damage significantly increased the anticonvulsant activity and the duration of action of both drugs. Nephrectomy increased the anticonvulsant activity and the duration of action of Mysoline but not of phenobarbital. The results suggest that, in the rat, both the liver and the kidney are important for the degradation and elimination of Mysoline, whereas the liver is the principal organ for the degradation of phenobarbital into products devoid of anticonvulsant activity.

IN A RECENT PUBLICATION (1) on the anticonvulsant properties of Mysoline (5-phenyl-5-ethyl-hexahydropyrimidine-4,6-dione), we called attention to the crystalluria which occurred in rats after moderate doses of the drug and in mice after comparatively large doses. Bogue and Carrington (2) have identified the crystals in rat urine as phenylethylmalondiamide. Because of our preliminary finding that Mysoline is more potent as an anticonvulsant in nephrectomized than in normal rats, the possibility was entertained that phenylethylmalondiamide was an active metabolic derivative to which Mysoline was converted in the body. However, phenylethylmalondiamide[1] was tested as an anticonvulsant and proved to be much less potent and more toxic than Mysoline (1). To explore further the metabolic fate of Mysoline, and since suitable chemical methods of analysis are not available, it was thought important to study the effect of nephrectomy and of liver damage on the potency and the duration of anticonvulsant action of the drug. It was anticipated that such a study would contribute information on the relative importance of the kidney and the liver in the fate and the excretion of this compound. Because Mysoline is a close congener of phenobarbital (the oxygen on the carbon in position 2 of the pyrimidine ring of phenobarbital is replaced by two atoms of hydrogen), parallel experiments were also conducted on the latter agent. The results obtained provide the basis for this report.

METHODS

The procedures utilized were the same as those previously described by us (3–5) and need only be summarized here. They consist of measuring the anticonvulsant potency of 5-phenyl-5-ethyl-hexahydropyrimidine-4,6-dione (Mysoline)[2] and 5-ethyl-5-phenylbarbiturate sodium (phenobarbital sodium) by the maximal electroshock seizure (M. E. S.) test at two, six, and twelve hours after drug administration in normal, liver-damaged, and bilaterally nephrectomized male Sprague-Dawley rats; the end point employed was prevention of the hind leg tonic extensor component of seizures induced by 150 ma. of alternating current applied for 0.2 second via corneal electrodes. Liver injury and nephrectomy have been shown not to alter the pattern of maximal electroshock seizures at the times of testing (3). Mysoline and phenobarbital were suspended in 10% acacia solution and given orally. The drugs were administered at times selected to permit testing at the time of maximum liver damage (36–48 hours after a single subcutaneous dose of 2 cc./Kg. of a 50% solution of carbon tetrachloride in peanut oil) or twelve hours after nephrectomy. For the determination of the ED_{50}, groups of at least eight rats were given various doses of anticonvulsant and tested at the times indicated above until at least three points were established between complete protection and no protection. The results obtained were then plotted on logarithmic probability paper and a regression line fitted to the plotted points by eye. From the plot of the data, the ED_{50} was determined and the 95% confidence limits were calculated by the method of Litchfield and Wilcoxon (6). The ED_{50} were then compared for the effects of the experimental procedures on the anticonvulsant potency and the duration of action of the drug.

Results.—The effects of liver damage and of nephrectomy on the anticonvulsant potency of Mysoline are graphically illustrated in Fig. 1, from which it can be seen that *liver damage* reduces the dose of Mysoline required to prevent the tonic extensor component of maximal electroshock seizures in 50% of rats. The reduction however is sig-

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[1] Kindly supplied by Dr. Glenn Ullyot, Smith, Kline & French Laboratories.

[2] Kindly supplied by Dr. John B. Jewell, Ayerst, McKenna & Harrison, Ltd., by arrangement with Imperial Chemical Industries Limited, England.

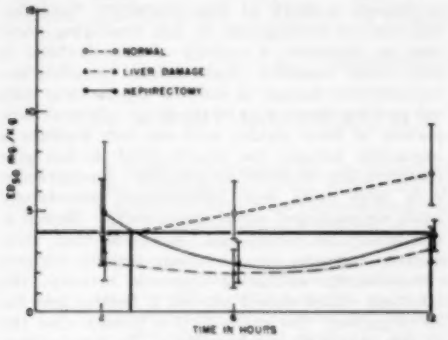

Fig. 1.—The effect of carbon tetrachloride-induced liver damage and of nephrectomy on the anticonvulsant activity and the duration of action of Mysoline. The anticonvulsant doses (ED_{50}) are expressed in mg./Kg. on the ordinate and the time intervals at which tests were made after drug administration are shown on the abscissa. The circles indicate the ED_{50} for normal rats; the solid triangles, the ED_{50} for liver-damaged rats; the dots, ED_{50} for nephrectomized rats. The 95% confidence limits are shown by the vertical bracketed lines.

nificant only for the six- and twelve-hour tests. The most striking increase in anticonvulsant activity was observed at the sixth hour after drug administration. For example, a dose of 4.9 mg./Kg. of Mysoline was required to protect 50% of normal rats, whereas only 2.0 mg./Kg. was required to produce the same level of protection in rats with damaged livers [ratio of ED_{50} and 95% confidence limits = 2.45 (1.41 to 4.26)].

Nephrectomy had a similar effect on the anticonvulsant potency of Mysoline. Thus, although there was no significant difference in anticonvulsant activity at two hours, a marked increase in potency of Mysoline was observed in the six- and twelve-hour determinations [e. g., ratio of ED_{50} in six-hour test and 95% confidence limits = 2.04 (1.20 to 3.47)] (Fig. 1).

With regard to the *duration* of anticonvulsant action of Mysoline, pertinent information was obtained by connecting the plotted points with smooth curves as shown in Fig. 1. The horizontal line, as shown in the figure, is usually drawn from the highest point on the ordinate which will permit the line to intercept all three dose-time curves. When this is not possible, it is then drawn so as to intercept the dose-time curve for normal animals and at least one of the dose-time curves for the experimental animals. Where the horizontal line intercepts the dose-time curves, vertical lines were drawn to the abscissa to indicate the duration of action (Figs. 1 and 2). It may be seen from Fig. 1 that both liver damage and nephrectomy prolong the anticonvulsant action of Mysoline. For example, a dose of 4 mg./Kg. of Mysoline protects 50% of normal rats for approximately three hours, whereas this dose provides the same level of protection for approximately twelve hours in liver-damaged or nephrectomized rats.

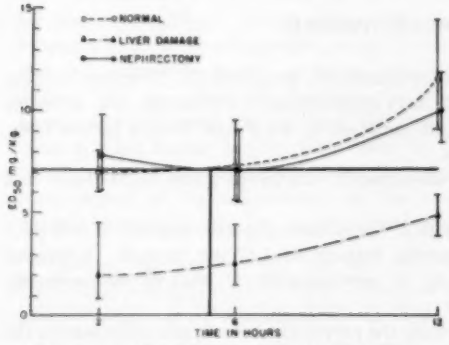

Fig. 2.—The effect of carbon tetrachloride-induced liver damage and of nephrectomy on the anticonvulsant activity and the duration of action of phenobarbital. See legend to Fig. 1 for description.

The results of parallel studies on phenobarbital are shown in Fig. 2. With regard to anticonvulsant *potency*, it may be seen from the figure that liver damage produces a marked and significant increase in the anticonvulsant activity of phenobarbital at all times at which determinations were made. For example, the ED_{50} for normal rats at six hours was 7.3 mg./Kg. as compared with 2.6 mg./Kg. for liver-damaged rats [ratio of ED_{50} and 95% confidence limits = 2.81 (1.53 to 5.23)]. In contrast, nephrectomy has no statistically significant effect on the anticonvulsant potency of phenobarbital as measured by the M. E. S. test.

The effect of the experimental procedures on the *duration* of anticonvulsant action of phenobarbital is illustrated in Fig. 2, which shows that a dose of 7.2 mg./Kg. will protect 50% of normal or nephrectomized rats for approximately five hours. However, the effect of liver damage on the duration of action of phenobarbital is so prominent that the exact magnitude could not be determined from the plot shown in Fig. 2. Nevertheless, it is obvious that the dose of 7.2 mg./Kg. of phenobarbital protects 50% of liver-damaged rats for more than twelve hours.

DISCUSSION

The data presented indicate that carbon tetrachloride-induced liver damage increases the anticonvulsant potency of both Mysoline and phenobarbital. The average increase in the intensity of anticonvulsant activity was 50.7% for Mysoline and 63.7% for phenobarbital. Nephrectomy markedly increases the anticonvulsant potency of Mysoline; the average increase was 47.9%. In contrast, nephrectomy has no significant effect on the anticonvulsant activity of phenobarbital within the time limits of these experiments. Liver damage produces a four-fold increase in the duration of anticonvulsant action of Mysoline and approximately a three-fold increase in that of phenobarbital. On the other hand, nephrectomy prolongs the anticonvulsant action of Mysoline four-fold, but it has no significant effect on that of phenobarbital.

The fact that liver damage increases both the anticonvulsant potency and duration of action of Mysoline and phenobarbital can be interpreted to mean that the liver is important for the degradation of these drugs to less potent substances or to substances devoid of anticonvulsant activity.

The finding that nephrectomy increases both the anticonvulsant potency and the duration of action of Mysoline suggests that the kidney is important for the elimination and/or the degradation of this compound or of any active product into which it may be converted. In marked contrast, nephrectomy has no significant effect on either the anticonvulsant potency or the duration of action of phenobarbital.

The only degradation product of Mysoline thus far identified is phenylethylmalondiamide (2). This substance appears as crystals in the urine of rats and mice given Mysoline. It is derived from Mysoline in the body by the opening of the pyrimidine ring and the loss of the internitrogen bridge. The liver is quite likely the major site of conversion of Mysoline to phenylethylmalondiamide. The enhanced activity of Mysoline observed after liver damage may be due in part to interference with this conversion, since we have found that phenylethylmalondiamide is very much less potent than Mysoline (7). For the same reason, the enhanced activity of Mysoline observed after nephrectomy cannot be due to interference with the excretion of phenylethylmalondiamide. Whether the anticonvulsant effects of Mysoline are due to the drug itself or some active metabolic conversion product(s) is unknown. In any event the liver and the kidney appear to be concerned in the degradation and the excretion of Mysoline and/or its active metabolite(s) in rats; should this situation be found to obtain in man, caution must then be exercised in the use of Mysoline in patients with liver injury or renal disease.

The finding that nephrectomy does not significantly increase the anticonvulsant activity of phenobarbital is in keeping with the well-established fact that only 15–25% of this drug is eliminated via the urine in the mammalian organism. The remainder of the phenobarbital is degraded in a manner and at loci yet to be determined (8). It is quite likely, however, that the liver plays a major role in this degradation. This would explain our finding that liver damage in rats increases and prolongs the anticonvulsant activity of phenobarbital. Nevertheless, certain investigators (9, 10), employing sleeptime as the index of activity of phenobarbital in rats, have reported that carbon tetrachloride-induced liver damage or subtotal hepatectomy does not prolong the activity of the drug. Critical comparison of these results with our own findings is impossible because the reports cited do not give complete data or statistical analysis. Furthermore, very large doses were administered parenterally while we employed small doses orally. Should it eventually be established, however, that liver damage enhances anticonvulsant activity without concomitantly enhancing hypnotic activity, the questions raised should provide a fruitful field for investigation. For example, it is possible that the as yet unidentified metabolite(s) of phenobarbital may have a different ratio of anticonvulsant to hypnotic potency than does phenobarbital itself. Again, it is possible that liver damage results in biochemical changes in the body, particularly in the brain, which act synergistically with the anticonvulsant effect but not with the hypnotic effect of phenobarbital. As long as exact information on the metabolic fate of phenobarbital is not available, further speculation is unwarranted.

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Radioactive Compounds Available

Limited supplies of radioactive corticosterone (Compound B), pregnane-3,11,20-trione, 3α,17α-dihydroxypregnane,11,20-dione, and 17α,hydroxy-21-acetoxypregnane,3,11,20-trione, are available to qualified investigators, free of charge, through funds supplied by the Public Health Service's National Institute of Arthritis and Metabolic Diseases.

In addition, microcurie samples of 3α,17α-dihydroxy-21-acetoxypregnane,11,20-dione and 3α,acetoxypregnane,11,20-dione are also available.

These compounds are all labeled at position 4 with C^{14}, and have a specific activity of 0.49 mc./m.mole, except the corticosterone, which has a specific activity of 1.47 mc./m.mole. Cortisone acetate, identically labeled, having a specific activity of approximately $^1/_3$ that of the previously announced cortisone acetate, may also be obtained.

Requests, in the form of a letter concisely describing the proposed research, should be sent to the Endocrinology Study Section, Division of Research Grants, National Institutes of Health, Bethesda 14, Maryland. Transfer and use of these materials are subject to current Atomic Energy Commission regulations.

The Effect of Phase Volume Ratio on Emulsion Type*

I. Beeswax-Borax Ointments

By RUPERT SALISBURY, E. E. LEUALLEN,† and L. T. CHAVKIN‡

A systematic method for the investigation of three component systems is used for the investigation of the system beeswax-borax, liquid petrolatum, and water, in an effort to determine the emulsion type of beeswax-borax ointments. In the event that a beeswax-borax emulsifier is capable of forming both oil in water and water in oil emulsions the component ratios at the point of change in emulsion type will be determined. Investigation of the emulsion type of a basic cold cream consisting of beeswax-borax, liquid petrolatum, and water shows that the resulting ointment may be either oil in water or water in oil. It has been established that in this particular system the ointment will be water in oil if the aqueous phase of the ointment is less than 45 per cent. If the aqueous phase is 45 per cent or more, the ointment will be of the oil in water type.

IN SPITE OF the long history of beeswax-borax type cold creams and the wide usage they enjoy, a great deal of confusion exists concerning the emulsion type they represent. Conflicting statements are found not only in the periodical literature (1–4) but in text and reference books as well (5–7). It is the purpose of this investigation to determine the emulsion types of basic cold creams made with all possible combinations of beeswax-borax, liquid petrolatum, and water. If the effect of variation of the phase volume ratio is to change the emulsion type, the proportions of the ingredients at the point where the emulsion type changes from oil in water to water in oil will be determined.

Using increments of 8.3 per cent in a triangular coordinate system, Schmalfuss, *et al.* (8), established a method of investigation for a three-component system in which an extremely good picture of the variations of the three components could be obtained from a total of 91 mixtures. Since this takes into consideration the three pure components as three points of investigation, and also considers possible mixtures of just two of the three components, it was considered too detailed for this investigation. Instead, using only combinations of all three components, it was decided that 55 ointments would give an adequate picture (Fig. 1). It is not necessary to compound all 55 mixtures for a general picture, instead a quick survey can be made with the 19 broad survey mixtures (circled in Fig. 1). The compositions of these mixtures can be read directly from the graph (Fig. 1), or from the listing in Table I. From the broad survey the area of interest can be located and a closer survey can be made in this region alone, but its closeness is not limited by the points used in the 55-mixture survey. Points of interest may be located on the graph and these combinations investigated so that areas in which the combinations have a certain desired quality (stability, color, consistency, gloss, etc.) can be very closely located.

This method lends itself to the investigation of any three-component system. It enables the investigator to locate the exact combination which best exhibits the desired results. This method should prove to be valuable in pharmaceutical investigations seeking to establish conditions for optimum suspension, emulsification—in fact, any chemical or physical quality desired for any three-component system.

EXPERIMENTAL

Materials used.—*White wax.* Tower brand, sun bleached white beeswax, m.p. 62.5-65.5°, acid value 17.2, ester value, 72.6.

Borax. Baker Chemical Co. sodium borate C. P Lot 82340. The amount of borax used was 5.8% of the wax required. This represents a stoichiometric amount based upon the acid value of the wax used.

Liquid Petrolatum. Plymouth brand, extra heavy, viscosity 340.

Distilled Water U. S. P. XIV.

Experimental Procedure.—The white wax was dissolved in the liquid petrolatum with the aid of heat. The borax was dissolved in the water and both solutions were heated to 80°. The aqueous phase was then poured into the oil phase while stirring with a Lab Mix stirrer (Chemical Publishing Company) at about 1500 r. p. m. The mixture was allowed to cool to 30° at the rate of approximately 2 degrees a minute, with constant stirring. All ointments were compounded on the basis of 100 Gm. total weight.

The resulting ointments were then tested for emulsion type by the dye method suggested by Clayton

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TABLE I.—SURVEY COMBINATIONS FOR A THREE-COMPONENT SYSTEM

Broad Survey	Close Survey	% A	% B	% C
1	1	8.3	8.3	83.3
	2	8.3	16.6	75.0
	3	8.3	25.0	66.6
2	4	8.3	33.3	58.3
	5	8.3	41.6	50.0
	6	8.3	50.0	41.6
3	7	8.3	58.3	33.3
	8	8.3	66.6	25.0
	9	8.3	75.0	16.6
4	10	8.3	83.3	8.3
	11	16.6	8.3	75.0
5	12	16.6	16.6	66.6
	13	16.6	25.0	58.3
	14	16.6	33.3	50.0
6	15	16.6	41.6	41.6
	16	16.6	50.0	33.3
	17	16.6	58.3	25.0
7	18	16.6	66.6	16.6
	19	16.6	75.0	8.3
	20	25.0	8.3	66.6
	21	25.0	16.6	58.3
8	22	25.0	25.0	50.0
	23	25.0	33.3	41.6
	24	25.0	41.6	33.3
9	25	25.0	50.0	25.0
	26	25.0	58.3	16.6
	27	25.0	66.6	8.3
10	28	33.3	8.3	58.3
	29	33.3	16.6	50.0
	30	33.3	25.0	41.6
11	31	33.3	33.3	33.3
	32	33.3	41.6	25.0
	33	33.3	50.0	16.6
12	34	33.3	58.3	8.3
	35	41.6	8.3	50.0
13	36	41.6	16.6	41.6
	37	41.6	25.0	33.3
	38	41.6	33.3	25.0
14	39	41.6	41.6	16.6
	40	41.6	50.0	8.3
	41	50.0	8.3	41.6
	42	50.0	16.6	33.3
15	43	50.0	25.0	25.0
	44	50.0	33.3	16.6
	45	50.0	41.6	8.3
16	46	58.3	8.3	33.3
	47	58.3	16.6	25.0
	48	58.3	25.0	16.6
17	49	58.3	33.3	8.3
	50	66.6	8.3	25.0
18	51	66.6	16.6	16.6
	52	66.6	25.0	8.3
	53	75.0	8.3	16.6
	54	75.0	16.6	8.3
19	55	83.3	8.3	8.3

(9). A few grains of a red, oil-soluble dye (Sudan III) were sprinkled upon a smear of the ointment. If the oil was in the external phase, then it was possible to see the dye diffusing. For convenience a 10× lens was used to observe the action of the dye. A confirmatory test was made with the water-soluble dye methyl orange. The results are tabulated in Table II and shown in Fig. 2.

Discussion.—The solubility of sodium borate in water is 1:16. For this reason it was not possible to compound ointments containing more than approximately 50% wax since the amounts of water in the combinations were not sufficient to dissolve the sodium borate necessary to saponify the amounts of cerotic acid present. Thus, only approximately one-half the number of possible combinations could be investigated.

The possibility of compounding ointments beyond this limiting solubility line was considered, but since no satisfactory procedure could be evolved the attempt was abandoned.

Results.—It would appear that the effect of variation of component ratio is capable of reversing the emulsion type of the basic cold cream investigated. The critical composition is in the vicinity

TABLE II.—EMULSION TYPES OF COLD CREAMS INVESTIGATED

Close Survey Number	Borax Used, Gm.	Wax Used, Gm.	Oil Used, Gm.	Water Used, Gm.	Emulsion Type Sudan III
1	0.48	8.3	8.3	83.3	O/W
2	0.48	8.3	16.6	75.0	O/W
3	0.48	8.3	25.0	66.6	O/W
4	0.48	8.3	33.3	58.3	O/W
5	0.48	8.3	41.6	50.0	O/W
6	0.48	8.3	50.0	41.6	W/O
7	0.48	8.3	58.3	33.3	W/O
8	0.48	8.3	66.6	25.0	W/O
9	0.48	8.3	75.0	16.6	W/O
10	0.48	8.3	83.3	8.3	W/O
11	0.96	16.6	8.3	75.0	O/W
12	0.96	16.6	16.6	66.6	O/W
13	0.96	16.6	25.0	58.3	O/W
14	0.96	16.6	33.3	50.0	O/W
15	0.96	16.6	41.6	41.6	W/O
16	0.96	16.6	50.0	33.3	W/O
17	0.96	16.6	58.3	25.0	W/O
18	0.96	16.6	66.6	16.6	W/O
19	0.96	16.6	75.0	8.3	Not enough water to dissolve borax
20	1.45	25.0	8.3	66.6	O/W
21	1.45	25.0	16.6	58.3	O/W
22	1.45	25.0	25.0	50.0	O/W
23	1.45	25.0	33.3	41.6	W/O
24	1.45	25.0	41.6	33.3	W/O
25	1.45	25.0	50.0	25.0	W/O
26	1.45	25.0	58.3	16.6	Not enough water to dissolve borax
27	1.45	25.0	66.6	8.3	
28	1.93	33.3	8.3	58.3	O/W
29	1.93	33.3	16.6	50.0	O/W
30	1.93	33.3	25.0	41.6	W/O
31	1.93	33.3	33.3	33.3	W/O
32	1.93	33.3	41.6	25.0	Not enough water to dissolve borax
33	1.93	33.3	50.0	16.6	
34	1.93	33.3	58.3	8.3	
35	2.41	41.6	8.3	50.0	O/W
36	2.41	41.6	16.6	41.6	O/W

No further ointment combinations possible since the amount of water present would not be sufficient to dissolve the required amount of borax.

Special Ointments Compounded to Define the Line More Sharply Between Oil in Water and Water in Oil.

A	0.29	5.0	50.0	45.0	O/W
B	0.58	10.0	45.0	45.0	O/W
C	0.87	15.0	40.0	45.0	O/W
D	1.16	20.0	35.0	45.0	O/W
E	1.45	25.0	30.0	45.0	O/W
F	1.74	30.0	25.0	45.0	O/W
G	2.03	35.0	20.0	45.0	O/W
H	2.32	40.0	15.0	45.0	O/W
I	2.61	45.0	10.0	45.0	O/W
J	2.03	35.0	30.0	35.0	W/O
K	2.15	37.0	28.0	35.0	W/O
L	2.15	37.0	25.0	38.0	W/O
M	2.32	40.0	22.0	38.0	W/O

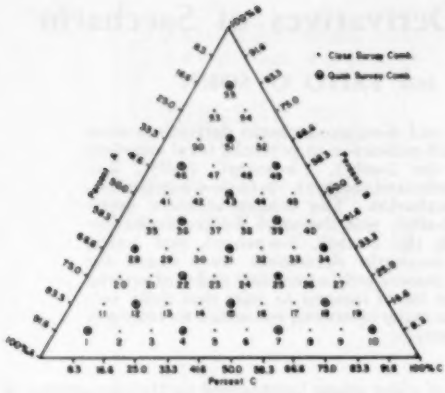

Fig. 1.—Survey of 55 ointments.

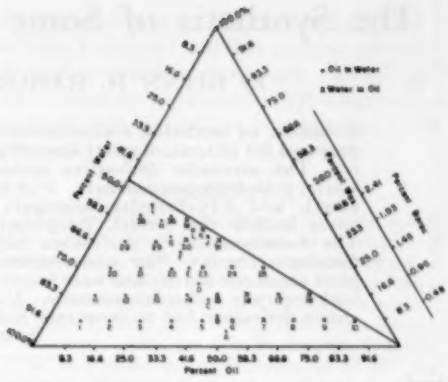

Fig. 2.—Test of ointments for emulsion type.

where water is present in a concentration of approximately 45%. If the aqueous phase is less than 45% the ointment will be of the water in oil emulsion type. If the aqueous phase is 45% or more, then the ointment will be of the oil in water type (Fig. 2).

An exception to this statement is found in ointment number 36 (Fig. 2). This ointment, although it contains only 41.6% water is of the oil in water type. It is difficult to account for this apparent anomaly due to the limitations set upon the investigation by the solubility of borax in water.

All ointments prepared were stable for a three-month period although those made with large percentages of oil (75% or more) showed a tendency to bleed water.

The fact that beeswax-borax does act as a weak emulsifier and, therefore, the emulsion type of the ointment formed is dependent upon the phase volume ratio, would serve to explain the confusion existing in the literature regarding the emulsion type of the liquid petrolatum, beeswax-borax cold creams. The confusion may well be due to the comparison of cold creams of different compositions and therefore of different emulsion types.

SUMMARY

1. A systematic method for the investigation of three-component systems has been used for the investigation of the system beeswax-borax, liquid petrolatum, and water.

2. Investigation of the emulsion type of a basic cold cream consisting of beeswax-borax, liquid petrolatum, and water shows that the resulting ointment may be either oil in water or water in oil depending upon the component ratio.

3. It has been established that in this particular system the ointment will be water in oil if the water content of the ointment is less than 45%. If the water content is 45% or more, the ointment will be oil in water.

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The Synthesis of Some Derivatives of Saccharin*

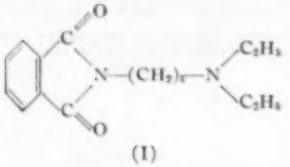
By GLENN H. HAMOR† and TAITO O. SOINE

A number of saccharin, 6-nitrosaccharin, and 6-aminosaccharin derivatives were prepared for pharmacological screening and evaluation as potential local anesthetics. The saccharin derivatives include the 2-ethyl, 2-*n*-propyl, 2-allyl, 2-*n*-amyl, 2-(β-diethylaminoethyl), 2-(β-dimethylaminoethyl), 2-(β-di-*n*-butylaminoethyl), and 2-(γ-diethylaminopropyl) saccharins. The 6-nitrosaccharin derivatives include the 2-ethyl, 2-*n*-propyl, 2-allyl, and 2-*n*-amyl 6-nitrosaccharins. The 6-aminosaccharin derivatives include the 2-ethyl, 2-*n*-propyl, and 2-allyl 6-aminosaccharins. The nonsubstituted saccharin derivatives were tested for local anesthetic activity and were found to possess only a very low order of activity both topically and subcutaneously. It may be of interest to note that these saccharin derivatives had no sweet taste and, in many instances, possessed an intensely bitter taste.

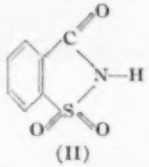
THE GREAT MAJORITY of compounds possessing local anesthetic activity have been esters of benzoic or substituted benzoic acids, but there are numerous compounds of different structure which are also endowed with activity. Among these are a number in which amide or imide structures replace the usual ester linkage. Indeed, an amide or imide may be considered as having —NH— substituted for the —O— of an ester and could be called "ammono" ester (1, 2). Representative of the amide type of local anesthetic are nupercaine and lidocaine.

Moore and Rapala (3) prepared a series of N-alkamine substituted derivatives of phthalimide. Most of their compounds possessed local anesthetic properties similar to that of procaine, with 1-(γ-diethylaminopropyl)phthalimide (I) being the best of the series from the standpoint of efficiency, toxicity, and lack of irritation.

Sulfur may also be introduced into the ester linkage, as evidenced by esters of sulfonic acids (4), dithiobenzoic acids (5), and *p*-aminothiobenzoic acids (6, 7) which all have some degree of local anesthetic activity.

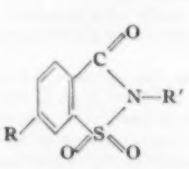


(I)



(II)

Because the imide structure is compatible with local anesthetic activity and, because the presence of sulfur in the molecule does not seem to preclude activity, it was thought that compounds of value might be obtained by the preparation of derivatives of benzoic sulfimide (II). Specifically, the compounds represented by formula III (see Tables I, II, and III for specific compounds) were prepared. Although the 2-alkyl derivatives of 6-nitro and 6-amino saccharin offered no difficulties it was found that the 2-dialkylaminoalkyl derivatives were not stable and suffered scission of the imide linkage with simultaneous addition of the alcohol, which was used as a reaction medium or crystallizing medium. Certain of the alkyl saccharins had been synthesized



R	R′
—NO_2	—CH_2CH_3
—NH_2	—*n*-C_3H_7
—H	—$CH_2CH{=}CH_2$
	—*n*-C_5H_{11}
	—$(CH_2)_2N(CH_3)_2$
	—$(CH_2)_2N(C_2H_5)_2$
	—$(CH_2)_2N(n\text{-}C_4H_9)_2$
	—$(CH_2)_3N(C_2H_5)_2$

(III)

earlier by others (8, 9) but had not been tested for pharmacological activity.

The saccharin derivatives were prepared by two methods. Procedure *A* involved refluxing a solution of sodium saccharin or sodium (or potassium) 6-nitro saccharin with the alkyl or dialkylaminoalkyl halide. The reaction solvents used were a carbitol-water mixture in the case of the alkyl derivatives and isopropyl alcohol for the dialkylaminoalkyl compounds. The second procedure, Procedure *B*, was that of Braun, Heider, and Müller (10), which consisted of the fusion or simple heating together of the free base dialkylaminoalkyl chloride with sodium saccharin or potassium 6-nitrosaccharin. The procedures are illustrated in the experimental section by specific examples and the compounds thus prepared are listed in Tables I–III. 6-Nitrosaccharin was prepared by the method of Noyes (11) which consists of the alkaline permanganate oxidation of 4-nitrotoluene-2-sulfonamide.

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TABLE I.—SACCHARIN DERIVATIVES

R	Formula	M. P., °C.	Recrystallizing Solvent	Procedure	Yield, %	Reaction Medium	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
$-C_2H_5$	$C_9H_9NO_3S$	95.5–96.5[a]	Ethanol (95%)[d]	*A*	82	Carbitol-H_2O	51.17	51.45	4.29	4.33
$-n\text{-}C_3H_7$	$C_{10}H_{11}NO_3S$	79–80[b]	Ethanol-H_2O	*A*	87	Carbitol-H_2O	53.31	53.74	4.92	4.97
$-CH_2CH{=}CH_2$	$C_{10}H_9NO_3S$	91–93[c]	Ethanol	*A*	90	Carbitol-H_2O	53.80	54.06	4.06	4.17
$-n\text{-}C_4H_{11}$	$C_{12}H_{15}NO_3S$	60.5–62[a]	Ethanol-H_2O	*A*	80	Carbitol-H_2O	56.89	57.44	5.97	6.17
$-(CH_2)_2N(CH_3)_2$	$C_{11}H_{14}N_2O_3S$	107–109	Ether-pet. ether[e]	*A*	14	2-Propanol	51.95	52.00	5.55	5.78
$-(CH_2)_2N(CH_3)_2{\cdot}HCl$	$C_{11}H_{15}N_2O_3SCl$	253.5–4.5 dec.	Ethanol	..	..	....	45.44	45.31	5.20	5.25
$-(CH_2)_2N(C_2H_5)_2$	$C_{13}H_{18}N_2O_3S$	65.5–68.5	Ether-pet. ether	*A*	24	2-Propanol	55.29	55.81	6.42	6.76
				B	27	....				
$-(CH_2)_2N(C_2H_5)_2{\cdot}HCl$	$C_{13}H_{19}N_2O_3SCl$	213–215	Ethanol	..	..	....	48.97	49.27	6.01	6.13
$-(CH_2)_2N(n\text{-}C_4H_9)_2{\cdot}HCl$	$C_{17}H_{27}N_2O_3SCl$	126.5–128.5	Absol. ethanol-ether	*B*	53	....	54.46	54.88	7.26	7.52
$-(CH_2)_3N(C_2H_5)_2{\cdot}HCl$[f]	$C_{14}H_{21}N_2O_3SCl$	167.5–169	2-Propanol	*A*	60	2-Propanol	50.52	50.38	6.36	6.61
				B	67	....	....	....	....	....

[a] Reported m. p. 94° (8). [b] Reported m. p. 74° (8). [c] Reported m. p. 98° (8). [d] Reported m. p. 58° (8). [e] The free base was a liquid, b. p. 205–209° (5 mm.). [f] "Ethanol" is 95% ethyl alcohol, U. S. P. [g] B. p. 30–60°.

Pharmacological tests[1] on the compounds listed in Table I indicated that they possess a low order of local anesthetic activity both topically and subcutaneously. The simple alkyl derivatives of saccharin were, in addition, very irritating to the guinea-pig skin.

EXPERIMENTAL[2]

2-Ethylsaccharin.—*Procedure A.*—Ethyl iodide (15.6 Gm., 0.1 mole) was added to a solution of sodium saccharin (20.5 Gm., 0.1 mole) in 50 ml. of carbitol and 8 ml. of distilled water. After refluxing and stirring for three hours, the cooled mixture was poured slowly with stirring into about 300 ml. of ice water. After one hour in the cold, 17.2 Gm. (82%) of a white material melting at 89–95° was collected. Recrystallization from ethanol raised the melting point to 95–96.5°. The literature reports 94° (8).

2-(β-Diethylaminoethyl)saccharin.—*Procedure B.*—Sodium saccharin (30 Gm., 0.12 mole) and β-diethylaminoethyl chloride (16.5 Gm., 0.12 mole) were mixed and heated to about 115° in an oil bath. Reaction took place immediately as evidenced by the development of a light yellow color and a quick rise in temperature to almost 130°. The mixture was then stirred and heated for an additional five minutes, cooled, and dissolved in a dilute hydrochloric acid solution. The acid solution was alkalinized with saturated sodium carbonate solution and the precipitated free base was thoroughly extracted with ether.[3] The ethereal extract was dried with anhydrous sodium sulfate and then evaporated to a small volume. Upon addition of petroleum ether (b. p. 30–60°) to turbidity followed by cooling, 2-(β-diethylaminoethyl) saccharin was obtained as 9.2 Gm. of white crystals, m. p. 61–64° (28%). Five recrystallizations from ether and petroleum ether raised the melting point to 65.5–68.5°.

2-(β-Diethylaminoethyl)saccharin Hydrochloride.—The hydrochloride was prepared by adding ethereal hydrogen chloride to a chilled absolute ethanol solution of the free base until no further precipitate was formed. Recrystallization gave a white crystalline bitter-tasting compound, m. p. 213–215°.

Of the dialkylaminoalkyl chlorides used, β-dimethylaminoethyl chloride hydrochloride and β-diethylaminoethyl chloride hydrochloride were obtained from commercial sources. β-Di-*n*-butylaminoethyl chloride hydrochloride and γ-diethylaminopropyl chloride hydrochloride were prepared by the method of Burckhalter, Stephens, and Hall (12). The free bases were obtained from the corresponding hydrochlorides by the procedure described in the above reference.

6-Nitro-2-*n*-propylsaccharin.—Procedure *A* was employed. Sodium 6-nitrosaccharin (6.2 Gm., 0.025 mole) prepared according to the method of Noyes

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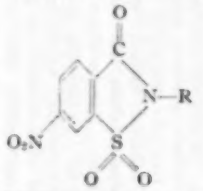
[1] The authors are indebted to the Eli Lilly Co., Indianapolis, Ind., for testing these compounds for local anesthetic activity.

[2] All melting points are uncorrected.

[3] An ether-insoluble compound was left behind at this point and because it was known that the free amine chlorides dimerize readily (12, 13) it was thought that the product might be 1,1,4,4-tetramethylpiperazinium dichloride.

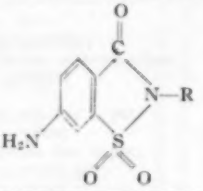
TABLE II.—6-NITROSACCHARIN DERIVATIVES



R	Formula	M. P., ° C.	Recrystallizing Solvent	Procedure	Yield, %	Reaction Medium	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
$-C_2H_5$	$C_9H_8N_2O_5S$	169–170[a]	Ethanol	*A*	50	Carbitol-H_2O	42.19	42.55	3.15	3.46
$-n$-C_3H_7	$C_{10}H_{10}N_2O_5S$	120–121	Ethanol	*A*	46	Carbitol-H_2O	44.44	44.78	3.73	3.92
$-CH_2CH{=}CH_2$	$C_{10}H_8N_2O_5S$	113–114.5	Ethanol	*A*	40	Carbitol-H_2O	44.77	44.90	3.01	3.30
$-n$-C_5H_{11}	$C_{12}H_{14}N_2O_5S$	98–99	Ethanol	*A*	48	Carbitol-H_2O	48.31	48.13	4.73	4.88

[a] Reported m. p. 172° (9).

TABLE III.—6-AMINOSACCHARIN DERIVATIVES



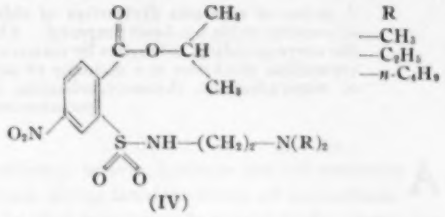
R	Formula	M. P., ° C.	Recrystallizing Solvent	Yield, %	Carbon, % Calcd.	Carbon, % Found	Hydrogen, % Calcd.	Hydrogen, % Found
$-C_2H_5$	$C_9H_{10}N_2O_3S$	215.5–216.5 dec.	Absolute ethanol	52	47.77	48.26	4.46	4.76
$-n$-C_3H_7	$C_{10}H_{12}N_2O_3S$	204–205 dec.	Absolute ethanol	96	49.98	50.22	5.04	5.26
$-CH_2CH{=}CH_2$[a]	$C_{10}H_{10}N_2O_3S$	228–230	Ethanol	56	50.41	50.51	4.23	4.48

[a] Obtained by reduction of the corresponding nitro compound with tin and hydrochloric acid (14). All others were reduced catalytically with hydrogen and 5% palladium-on-carbon at initial pressures of about 40 lbs. p. s.i.

(11) and *n*-propyl iodide (4.2 Gm., 0.025 mole) were refluxed for eight hours in a carbitol-water mixture to yield 3 Gm. of a solid melting at 118–119° (46%). Recrystallization from ethanol gave light yellow crystals, m. p. 120–121°.

6-Amino-2-*n*-propylsaccharin.—6-Nitro-2-*n*-propylsaccharin (3.2 Gm., 0.01 mole) in 200 ml. of absolute ethanol was reduced with hydrogen at room temperature at about three atmospheres pressure with 0.8 Gm. of 5% palladium-on-charcoal catalyst. From the evaporated filtrate 2.8 Gm. (96%) of a gray-green solid separated on cooling, m. p. 196–200°. Four recrystallizations raised the melting point to 204–205° (decomp.).

The synthesis of 2-dialkylaminoalkyl derivatives of 6-nitrosaccharin was attempted by the same methods used in the saccharin series. The resultant compounds were oils or, if solid, were compounds in which the analytical values obtained did not agree with those calculated for the desired compounds. One explanation offered for this behavior is that rupture of the sulfimide link between carbon and nitrogen has occurred with simultaneous addition of isopropyl alcohol (used either as a reaction medium or recrystallizing solvent) across the broken linkage. The analytical values obtained agree with the values calculated for compounds of the isopropyl 4-nitro-2-dialkylaminoalkylsulfonamidobenzoate type (IV).



(IV)

Upon reduction to the 6-amino compounds the analytical values obtained agreed with the values calculated for the esters rather than for the substituted saccharins.[4]

[4] The dimethylaminoethyl substituted product could not be recrystallized in a condition suitable for analysis.

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WHO MAKES IT?

The National Registry of Rare Chemicals, Armour Research Foundation of the Illinois Institute of Technology, 35 West 33rd Street, Chicago 16, Ill., seeks information on sources of supply for the following chemicals:

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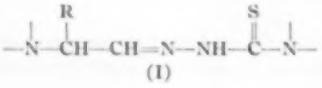
Ptyalin
3,5-Pyridinedicarboxylic acid
Lactic aldehyde
Glycollic aldehyde
N-Methylvaline
8-Hydroxy-1,2,3,4-tetrahydroquinoline
2,3-Dihydrofuran
Chloropromazine
β-Chloro-α-naphthol
1-Decanesulfonic acid
6-Methylindole
1,4-Dibromo-2-butyne
3,5-Dihydroxycinnamic acid
Pyrazine-2,3-dicarboxylic acid
4-Methyltryptophane
Glycyrrhetinic acid
Amygdalin
δ-Tocopherol
Ornithuric acid
Protoanemonin
N-Methyl-6(1)-pyridone-3-carboxamide
cis-Diphenylethylene oxide
4-Methoxyproline
1-Methylxanthine
Capsaicin
Cinchol
Kynurenine
l-Limonene
Gymnemic acid

Derivatives of *alpha* Amino Aldehydes*

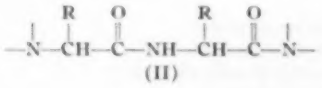
By WILLIAM O. FOYE and JOHN J. HEFFERREN†

A series of nitrogen derivatives of aldehyde analogs of some naturally occurring *dl* α-amino acids has been prepared. The conversion of the phthaloylamino acids to the corresponding aldehydes by means of the Rosenmund method of reduction gave crystalline aldehydes in a majority of cases. The aldehydes were readily converted to semicarbazone, thiosemicarbazone, and dithiobiuret derivatives for testing as possible antiviral agents.

ANDREWES (1) has outlined several possible mechanisms by which antiviral agents may act, among which is that of an antimetabolic effect caused by structures related to essential metabolites of the virus. That this effect may actually be operative has been indicated by the action of sulfonic acid analogs of naturally occurring α-amino acids (2), as well as by various aromatic aldehyde thiosemicarbazones (3), which have been shown to be highly active against a variety of viruses. This evidence suggested the possibility that aldehyde analogs of naturally occurring α-amino acids might be inhibitory to the multiplication of viruses, and a series of analogs of this type has accordingly been prepared. These compounds were converted to nitrogen derivatives (I) which provide a similarity to simple dipeptides (II), when it is considered that the nitrogen side chains are similar to glycine in molecular size and shape, and the aldimine linkage is analogous to a dehydrated amide linkage.



(I)



(II)

Literature on the previous preparation of stable aliphatic α-amino aldehydes is scanty. Fischer and Kametaka (4), however, obtained L-α-aminopropionaldehyde, aminoacetaldehyde, and *dl*-α-amino-α-phenylpropionaldehyde as the diethyl acetals by reduction of the amino acid esters with sodium amalgam. Akabori (5) and Bullerwell and Lawson (6) used the same procedure to obtain a number of amino aldehydes which, however, were not isolated, but converted to imidazoles.

Another method was employed by Radde (7) to obtain phthalimidoacetaldehyde and phthalimidopropionaldehyde—namely, a Rosenmund reduction of the α-phthaloylamino acid chloride. Balenović, *et al.* (8), have recently utilized this method to prepare optically active amino aldehydes from L-alanine, *S*-benzyl-L-cysteine, and *O*-methyl-L-tyrosine.

Other methods attempted in this laboratory for the preparation of α-amino aldehydes include the controlled oxidation of *N*-benzoylaminoethanol, the reduction of β-phthalimidopropionitrile, and the hydrolysis of phthalimidoacetaldehyde dimethyl acetal. These methods, as well as a Rosenmund reduction of α-benzoylamino acids, failed to give appreciable yields; the latter method failed because of solubility difficulties. Accordingly, the method of Radde was employed, which involves a Rosenmund reduction of the α-phthaloylamino acid chloride. This procedure was found to be successful with all α-amino acids tried except methionine, where the sulfur possibly reacts with the acid chloride, since the acid chloride obtained resisted reduction. The general procedure followed is illustrated by Eq. 1.

Although Radde claimed that this method was successful only in the case of α-amino acid chlorides, it was found that β-phthalimidopropionaldehyde could be prepared as successfully as any of the α-amino aldehydes. It was also found that use of more catalyst for the reduction than that usually required gave much better results. While Mosettig and Mozingo (9) specify one part of catalyst for five to ten parts of acid chloride, the reduction of the α-phthalimido acid chlorides proceeded much faster and gave purer aldehydes with one part of catalyst for two to three parts of acid chloride.

The amino aldehydes, in general, were isolated as the phthaloyl derivatives, which crystallized from the reaction mixture on addition of hydrocarbon solvents. The longer chain aldehydes,

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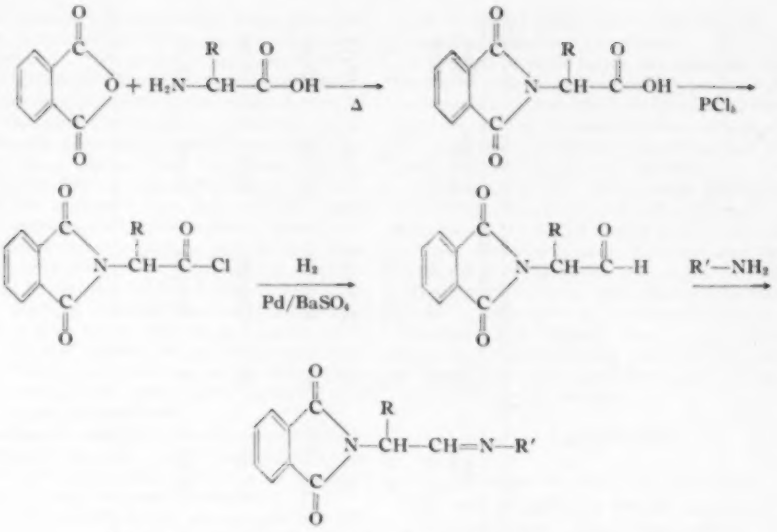
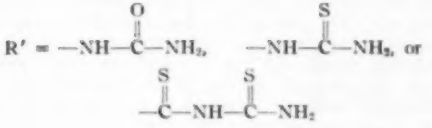
Abstracted from the dissertation of John J. Hefferren submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, University of Wisconsin, September, 1953.
† Fellow of the American Foundation for Pharmaceutical Education 1950–1953.



Equation 1.—Preparation of α-phthalimido aldehyde derivatives.



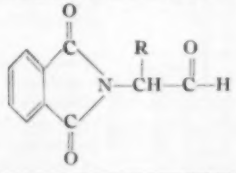
prepared from leucine and valine, could not be obtained in crystalline form. The oils which resulted in these cases were readily converted to crystalline aldehyde derivatives, however. The phthaloyl groups were allowed to remain in the final products for the sake of greater stability.

The physical constants and analytical results of the α-phthalimido aldehydes prepared are recorded in Table I and those of the aldehyde derivatives in Table II.

EXPERIMENTAL

Phthalimido Acids.—The phthalimido acids were prepared by the general procedure of Reese (10), in which a finely ground mixture of the amino acid and phthalic anhydride is heated with stirring at 130–150°. The products were obtained by recrystalliza-

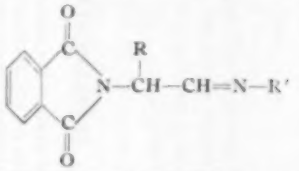
TABLE I.—PHTHALIMIDO ALDEHYDES FROM AMINO ACIDS



Amino Acid Used	Yield, % (From Phthalimido Acid)	M. P.[a] Found	M. P.[a] Recorded[b]	Analysis[c] Calcd.	Analysis[c] Found
Glycine	70	112–114°	113–114.5°		
dl-Alanine	51	109–111°	108.5–111°		
dl-β-Alanine	65	115–117°		C, 65.01 H, 4.46	C, 65.21 H, 4.79
dl-Phenylalanine	50	75–78°		C, 73.11 H, 4.69	C, 73.44 H, 4.91
dl-Leucine	Oil				
dl-Valine	Oil				

[a] All melting points are corrected.
[b] Radde (7).
[c] Analyses for C and H were carried out by the Clark Microanalytical Laboratory, Urbana, Illinois.

TABLE II.—PHTHALIMIDO ALDEHYDE DERIVATIVES



Aldehyde	Semicarbazone Yield, %	Semicarbazone M. P.,[a] °C.	Semicarbazone Nitrogen Calcd.	Semicarbazone Anal.[b] Found	Thiosemicarbazone Yield, %	Thiosemicarbazone M. P., °C.	Thiosemicarbazone Nitrogen Calcd.	Thiosemicarbazone Anal. Found	Dithiobiuret Derivatives Yield, %	Dithiobiuret Derivatives M. P., °C.	Dithiobiuret Derivatives Nitrogen Calcd.	Dithiobiuret Derivatives Anal. Found
Phthalimido-ethanal	85	224–226[c]	...	...	81	215–216	21.36	21.92	15	250–251	18.37	18.29
2-Phthalimido-propanal	85	224–225[d]	...	...	60	206–208	20.28	19.99	20	251–253	17.49	17.23
3-Phthalimido-propanal	66	219–220	21.53	21.30	70	211–212	20.28	19.67	20	243–244	17.49	17.12
2-Phthalimido-3-phenyl propanal	88	215–216	16.66	16.52	85	204–205	15.90	15.13	20	256–258	14.13	13.63
2-Phthalimido-4-methyl pentanal	90	200–201	18.53	18.28	65	190–191	17.60	17.27	18	258–260	15.54	15.22
2-Phthalimido-3-methyl butanal	70	228–229	19.44	18.67	74	214–215	18.41	17.21	14	237–239	16.08	15.75

[a] All melting points are corrected.
[b] Analyses for N were carried out by the Clark Microanalytical Laboratory, Urbana, Illinois.
[c] Radde (7) lists 233–244°.
[d] Radde (7) lists 225.5–226.5°.

tion from water or aqueous alcohol, although it was necessary to extract first with ether in the case of phthaloylvaline. The yields ranged from 85–95%.

Phthalimido Acid Chlorides.—The acid chlorides were all prepared in essentially the same manner by shaking the phthalimido acids (0.02–0.04 mole) with equimolar quantities of phosphorus pentachloride in a 50-cc. Claisen flask for fifteen minutes, followed by heating the mixtures on the steam bath for forty-five minutes. The light yellow liquids obtained were warmed under reduced pressure to remove phosphorus oxychloride, and the acid chlorides were crystallized from hydrocarbon solvents. In the case of phthaloylleucine and phthaloylvaline, however, the acid chlorides were obtained only as oils. The use of oxalyl chloride was necessary to prepare the acid chloride of phthaloylmethionine. Thionyl chloride gave no better results than phosphorus pentachloride, which gave yields of acid chlorides ranging from 85–95%.

Preparation of Catalyst.—The catalyst employed for reduction of the acid chlorides was palladium on barium sulfate (palladium content 5%) prepared according to the procedure of Mozingo (11).

The quinoline-sulfur poison or regulator was prepared according to the procedure of Hershberg and Cason (12), giving a xylene solution which contained 0.1 Gm. of regulator per cc.

Phthalimido Aldehydes.—The reduction of the acid chlorides followed essentially the original method of Rosenmund and Zetsche (13). The acid chloride (0.02–0.04 mole) was dissolved in three to ten times its weight of purified toluene (refluxed over sodium) in a 250-cc. three-necked flask equipped with a mercury-sealed stirrer, hydrogen trap, and condenser with tubing attached to permit the titration of the evolved gases. The catalyst was added to give a weight ratio of one part of catalyst to two or three parts of acid chloride, and the regulator was added in an amount one-tenth that of the catalyst.

After the mixture was thoroughly flushed with hydrogen, it was heated to reflux temperature and then stirred until 80–90% of the theoretical amount of hydrogen chloride was evolved, as determined by titration with 1.0 *N* sodium hydroxide. This generally required four to five hours, at which time the evolution of hydrogen chloride was extremely slow. Heating beyond five hours generally resulted in considerable decomposition.

Norite was then added, the catalyst was removed by filtration, and the resulting solution was chilled until crystals appeared. The addition of petroleum ether generally gave a second crop. After recrystallization from mixtures of benzene and petroleum ether, the phthalimido aldehydes were obtained in yields of 60–76%. The aldehydes prepared from phthaloylleucine and phthaloylvaline were obtained as light yellow oils which could not be crystallized.

Preparation of Aldehyde Derivatives.—The semicarbazones were prepared in the customary fashion by heating the aldehydes with semicarbazide hydrochloride and sodium acetate. The thiosemicarbazones were prepared by heating with thiosemicarbazide in aqueous alcohol.

The dithiobiuret derivatives were prepared by the procedure of Foye and Hefferren (14) in which dithiobiuret is refluxed with a slight excess of aldehyde in glacial acetic acid which contains a small amount of anhydrous zinc chloride. After refluxing for thirteen or more hours, the solutions were concentrated slightly and crystalline products were obtained, after at least two days standing at room temperature. After recrystallization from glacial acetic acid, the dithiobiuret condensation products were obtained in yields of 15–20%.

Treatment of the phthalimido aldehydes with phenylhydrazine resulted in phenylhydrazones with no cleavage of the phthaloyl group. Phthalimidoethanal phenylhydrazone was prepared from the aldehyde in 60% yield, and melted at 163–165°, which agrees with the value reported by Radde (7). However, treatment of phthalimidoethanal phenylhydrazone with hydrazine hydrate, according to the method of Ing and Manske (15) resulted in apparent decomposition, and a stable product could not be isolated from the reaction.

SUMMARY

1. A series of aldehyde analogs of α-amino acids was prepared to test for the possibility of antiviral action. Stable derivatives were obtained by preparing the α-phthalimido aldehydes which were converted to semicarbazone, thiosemicarbazone, and dithiobiuret derivatives.

2. A general method of preparation was found in the Rosenmund method of reduction of the α-phthalimido acid chlorides, except where sulfur was present in the molecule. A β-phthalimido aldehyde was also prepared by this method.

3. The following new compounds were characterized: 3-phthalimidopropanal, 2-phthalimido-3-phenylpropanal, the semicarbazone, thiosemicarbazone, and dithiobiuret derivatives of 3-phthalimidopropanal, 2-phthalimido-3-phenylpropanal, 2-phthalimido-4-methylpentanal, and 2-phthalimido-3-methylbutanal, and the thiosemicarbazone and dithiobiuret derivatives of phthalimidoethanal and 2-phthalimidopropanal.

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Book Notices

BOOKS RECEIVED

Infrared Absorption Spectra of Steroids—An Atlas. By KONRAD DOBRINER, E. R. KATZENELLENBOGEN, and R. NORMAN JONES. Interscience Publishers, Inc., New York, 1953. xiv pp. + 308 spectra. 16 x 24 cm. Price $11.50.

The Van Nostrand Chemist's Dictionary. Board of Editors: JURGEN M. HONIG, *et al.* D. Van Nostrand Co., Inc., New York, 1953. 761 pp. 16 x 24 cm. Price $10.

Biochemical Preparations. Vol. 3. ESMOND B. SNELL, Editor-in-Chief. John Wiley & Sons, Inc., New York, 1953. 128 pp. 15.5 x 23.5 cm. Price $3.50.

Chemical Methods in Industrial Hygiene. By FREDERICK H. GOLDMAN and MORRIS B. JACOBS. Interscience Manuals No. 3. Interscience Publishers, Inc., New York, 1953. 274 pp. 13 x 18.5 cm. Price $3.75.

A Policy for Scientific and Professional Manpower. By the National Manpower Council. Columbia University Press, New York, 1953. xvi + 263 pp. 16 x 24 cm. $4.50.

Sixth Annual Report of the Committee on Growth of the National Research Council, to the American Cancer Society, Inc., July, 1950–June, 1951. Division of Medical Sciences, National Research Council, Washington, D. C. 373 pp. Paper bound. 15 x 23 cm.

Second Report on Institutional Research Grants of the American Cancer Society, September, 1950–August, 1951. American Cancer Society, Inc., New York, N. Y., 1952. 233 pp. Paper bound. 15.5 x 23 cm.

Symptons of Visceral Disease—A Study of the Vegetative Nervous System in Its Relationship to Clinical Medicine. 7th ed. By FRANCES MARION POTTENGER. The C. V. Mosby Co., St. Louis, 1953. 446 pp. 14.5 x 22.5 cm. Illustrated. Price $7.50.

Pharmacognosy Laboratory Manual. By MAYNARD W. QUIMBY, RAYMOND W. VANDER WYK, and WILLIAM E. HASSAN, JR. (Division of Pharmacognosy and Allied Sciences, Department of Biological Sciences, Massachusetts College of Pharmacy, Boston.) Burgess Publishing Co., Minneapolis 15, Minn., 1953. 108 pp. 22 x 27.5 cm.

Smithsonian Institution—Annual Report of the Board of Regents. Publication 4111. U. S. Government Printing Office, Washington 25, D. C. 15.5 x 24 cm. 461 pp. Price $2.75.

Forty-First Annual Meeting, American Drug Manufacturers Association. Boca Raton Club, Boca Raton, Florida, April 13–16, 1953. Private Printing: The Lord Baltimore Press, Baltimore, Md., 1953. 189 pp. Illustrated. 16 x 24 cm.

Canada's Food and Drug Laws. By ROBERT E. CURRAN. Commerce Clearing House, Inc., New York, 1953. 1138 pp. 17.5 x 25.5 cm. Price $19.50.

The Book of Health. By R. LEE CLARK, JR., and RUSSELL W. CUMLEY, Editors-in-Chief. Elsevier Press Inc., New York, 1953. 768 pp. Illustrated. 19 x 26.5 cm. Price $10.

This book was written by two experienced authors of medical articles for lay readers, assisted by an editorial board of 242 associates, most of whom are physicians. They have succeeded in producing an interesting encyclopedic form of text which conveys a great deal of information while omitting detailed scientific explanations. One aim of the editors is to present sufficient information to the layman to enable him, when and if necessary, to be an informed patient and to know something about living to avoid being a patient. An intelligent layman should obtain information from this book that will help him fulfill the editors' aim.

The text is arranged according to the following chapter headings: Life Begins; The Child; The Body; Disease Producing Organisms; The Respiratory System; Blood and Blood-forming Organs; The Heart and Circulation; The Skin; the Skeleton and Muscles; The Endocrine System; The Brain and Nervous System; The Teeth; The Digestive System; The Urinary System; The Reproductive System; The Breast; The Eye; The Ear; The Mind; The Later Years; Nutrition; Sickness at Home; Physical Injuries and First Aid; Tropical Diseases; Sanitation; Medicine and the Law; Medical History; The Medical Profession.

The text contains many illustrations and pictures, some in color. A statistical appendix and an extensive index and glossary are included.

Aids to Qualitative Pharmaceutical Analysis. By J. PRIESTMAN and F. C. G. EDWARDS. Bailliere, Tindall and Cox, London, 1953. (The Williams & Wilkins Co., Baltimore, Md.) 144 pp. 10.5 x 16.5 cm. Price $2.

This pocket-size book is a compilation of elementary information pertaining to qualitative inorganic and organic tests. The text is intended as a review aid for students.

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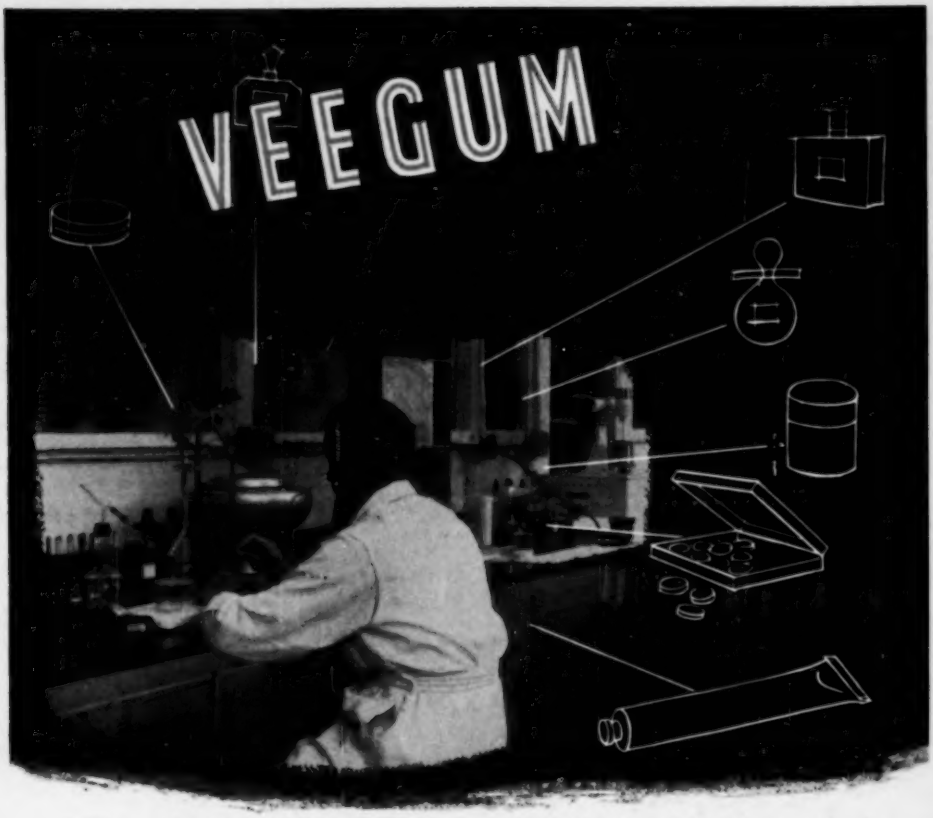

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